

**Tipos e concentrações de auxinas no enraizamento *ex vitro* de mirtilheiro
'Woodard'****Types and concentrations of auxins in the *ex vitro* rooting of 'Woodard'
mirtiller**

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RESUMO

Na micropropagação do mirtilheiro (*Vaccinium* sp.) a realização da etapa de enraizamento *ex vitro* (ao invés de *in vitro*) poderá representar uma alternativa para a redução dos custos de produção das mudas. Diante disso, o objetivo deste trabalho foi avaliar o efeito de concentrações das auxinas ácido indolbutírico (AIB) e ácido naftaleno-acético (ANA) no enraizamento *ex vitro* de mirtilheiro 'Woodard'. O delineamento experimental foi o inteiramente casualizado, em esquema bifatorial (dois tipos (AIB; ANA) e quatro concentrações (0; 0,2; 0,4 e 0,6 mg L⁻¹) **de auxina**), totalizando oito tratamentos e quatro repetições, sendo cada repetição constituída de uma bandeja Sanpack® com 15 microestacas. Aos 60 dias avaliou-se: sobrevivência, porcentagem de enraizamento, número de raízes, comprimento da maior raiz e massa de matéria seca das raízes. Para a sobrevivência, não houve influência da aplicação das auxinas, verificando-se alta eficiência (100%). Da mesma forma foi verificado para a porcentagem de enraizamento das microestacas. Para o número de raízes, a maior média foi observada no tratamento com o uso de AIB, quando utilizado a concentração 0,2 mg L⁻¹; não diferindo da concentração 0,6 mg L⁻¹. O comprimento da maior raiz, apresentou as maiores médias quanto utilizado AIB. Para a massa de matéria seca das raízes, o tratamento sem a utilização das auxinas apresentou a maior massa; entretanto, não diferiu dos tratamentos com a utilização de AIB. Assim, o enraizamento *ex vitro* de mirtilheiro 'Woodard' pode ser realizado sem o uso das auxinas ácido indolbutírico e ácido naftaleno-acético.

Palavras chaves: *Vaccinium* sp. Micropropagação. Regulador de Crescimento. Pequenas frutas.

ABSTRACT

In the blueberry (*Vaccinium* sp.) Micropropagation, the *ex vitro* rooting step (instead of *in vitro*) may represent an alternative to reduce seedling production costs. Therefore, the objective of this work was to evaluate the effect of concentrations of auxins indolbutyric acid (AIB) and naphthalene-acetic acid (ANA) on the *ex vitro* rooting of blueberry 'Woodard'. The experimental design was completely randomized, in a bifactorial scheme (two types (AIB; ANA) and four concentrations (0; 0.2; 0.4 and 0.6 mg L⁻¹) of auxin), totaling eight treatments and four repetitions, each repetition consisting of a Sanpack® tray with 15 micropiles. At 60 days it was evaluated: survival, percentage of rooting, number of roots, length of the largest root and root dry matter. For survival, there was no influence of the application of auxins, with high efficiency (100%). Likewise it was verified for the percentage of rooting of the microcuttings. For the number of roots, the highest average was observed in the treatment with the use of IBA, when using the concentration 0.2 mg L⁻¹; not differing from the 0.6 mg L⁻¹ concentration. The length of the largest root showed the highest averages when using IBA. For the dry matter mass of the roots, the treatment without the use of auxins showed the highest mass; however, it did not differ from treatments with the use of IBA. Thus, the *ex vitro* rooting of blueberry 'Woodard' can be performed without the use of auxins indolbutyric acid and naphthalene-acetic acid.

Keywords: *Vaccinium* sp. Micropropagation. Growth Regulator. Small fruits.

1 INTRODUÇÃO

Dentre as pequenas frutas o mirtilheiro (*Vaccinium* spp.) é uma das culturas mais promissoras para o cultivo na região sul do Brasil, devido às condições edafoclimáticas favoráveis à adaptação de várias cultivares, além de ser muito apreciado pelo sabor exótico e valor econômico, além da ampla divulgação dos frutos como fonte da longevidade (SHARPE, 1980; RUFATO; ANTUNES, 2016).

No Brasil essa cultura encontra-se em fase de desenvolvimento, ocasião em que se busca um sistema de produção eficiente e competitivo para inserir o país no rol dos grandes produtores mundiais (RUFATO; ANTUNES, 2016). Assim, um dos fatores primordiais para o sucesso no cultivo é a utilização de mudas de qualidade (OLIVEIRA, 2020).

Uma opção para aumentar a disponibilidade de mudas dessa frutífera é a micropropagação (DAMIANI; SCHUCH, 2009). Essa técnica possibilita, além da produção de grande quantidade de plantas em curto período de tempo, a obtenção de plantas livres de doenças e a propagação de espécies difíceis de serem multiplicadas por outros métodos (GRATTAPAGLIA; MACHADO, 1998). Por outro lado, implica em elevados custos na produção comercial de mudas (KODYM; ZAPATA-ARIAS, 1998).

Visando a redução nos custos da muda micropropagada, o enraizamento *ex vitro* das plântulas apresenta-se como alternativa, pois de acordo com Pedrotti e Voltolini (2001), propicia a diminuição de 50% do preço final da muda, comparativamente ao uso do enraizamento *in vitro*.

No enraizamento *ex vitro* as partes aéreas dos explantes são manipulados como microestacas e todo o processo de enraizamento é feito em substrato. Segundo Grattapaglia e Machado (1998), a regeneração de raízes diretamente no substrato produz um sistema radicular funcional e com maior número de raízes secundárias.

Para promover o enraizamento em estacas de espécies frutíferas auxinas como ácido indolbutírico e ácido naftaleno-acético em aplicação exógena mostram-se bastante eficientes (FACHINELLO et al., 2005). Porém, a resposta da planta à auxina varia tanto com a natureza do tecido, época do ano e condições fisiológicas da planta e da estaca, quanto com a concentração (XAVIER et al., 2009).

Alguns estudos com enraizamento *ex vitro* e uso de reguladores de crescimento foram realizados com amoreira-preta (*Rubus* sp.) 'Xavante' (PELIZZA et al., 2013); amoreira-preta 'Brazos' (AUGUSTO et al., 2006); porta-enxerto de macieira (*Malus pumilla*) M.9 (PEDROTTI; VOLTOLINI, 2001); abacaxizeiro (*Ananas comosus*) (DIAS et al., 2010); erva-mate (*Ilex paraguariensis* A. St Hil.) (TRONCO et al., 2015). Entretanto, são escassas as informações a respeito do uso desta técnica em mirtilheiro, o que ressalta a necessidade de estudos, que poderão contribuir com avanços na micropropagação dessa frutífera.

Nesse sentido, o presente trabalho teve como objetivo avaliar o efeito de diferentes concentrações das auxinas ácido indolbutírico e ácido naftaleno-acético no enraizamento *ex vitro* de mirtilheiro 'Woodard'.

2 METODOLOGIA

O experimento foi realizado no período de setembro a novembro de 2018, no Laboratório de Propagação de Plantas Frutíferas, pertencente ao do Departamento de Fitotecnia, da Faculdade de Agronomia Eliseu Maciel, da Universidade Federal de Pelotas (UFPel/RS), localizada no município Capão do Leão-RS.

O delineamento experimental foi o inteiramente casualizado, em esquema bifatorial, sendo os fatores: tipos de auxina (ácido indolbutírico (AIB) e ácido naftaleno-acético (ANA)) e concentrações (0; 0,2; 0,4 e 0,6 mg L⁻¹), totalizando oito tratamentos, com quatro repetições,

sendo cada repetição constituída de uma embalagem plástica transparente e articulada Sampack® (22x14x10 cm) com 15 microestacas cada.

Foram utilizadas microestacas provenientes do cultivo *in vitro* de mudas de mirtilheiro ‘Woodard’, medindo 2,5 de comprimento com sete gemas, sete folhas e ápice caulinar, mantidas em meio de cultura WPM (Wood Plant Media - LOYD; McCOWN, 1980). Aos 60 dias após a multiplicação *in vitro* as plantas foram retiradas dos frascos, lavadas em água corrente para a limpeza do meio de cultivo e, em seguida, o sistema radicular foi imerso nos reguladores de crescimento e concentrações, conforme o tratamento.

Em seguida, as microestacas foram acondicionadas nas embalagens transparentes e articuladas Sanpack®, contendo um litro vermiculita média expandida, sendo previamente hidratadas com 500 mL de água destilada. Após, foram mantidas em casa de vegetação com temperatura controlada a $25\pm 2^{\circ}\text{C}$ e luminosidade natural. Durante o enraizamento, sempre que necessário, procedeu-se a rega com 25 mL de água destilada, com o auxílio de um pulverizador manual Tramontina®, mantendo-se as embalagens fechadas para evitar a desidratação.

Após 60 dias as variáveis avaliadas foram: sobrevivência (%), enraizamento, número de raízes, comprimento da maior raiz (cm) e massa de matéria seca das raízes (g). Para o comprimento médio das raízes utilizou-se régua graduada. Quanto a obtenção da massa de matéria seca, o material foi mantido em estufa com ventilação forçada a 50°C até a obtenção de peso constante e, após, pesado em uma balança digital analítica.

Os dados foram submetidos à análise de variância e as médias comparadas pelo teste de Tukey a 5% de probabilidade.

3 RESULTADOS E DISCUSSÃO

Em relação a variável sobrevivência foi constatada 100%, ou seja, todos os explantes sobreviveram, independente do tratamento. Em trabalho realizado sobre o enraizamento *ex vitro* de mirtilheiro, Pelizza (2009) verificou resultados semelhantes a este trabalho, em que ‘Bluebelle’ e ‘Woodard’ apresentaram 99,4 e 100% de sobrevivência, respectivamente. Para Oliveira (2020), a porcentagem de sobrevivência pode interferir na rentabilidade do produtor, visto que está relacionada com a quantidade de mudas a ser comercializada.

Avaliando o enraizamento, não houve significância estatística entre os tipos de auxina e suas concentrações, verificando-se 100 %, ou seja, alta eficiência no enraizamento das microestacas de mirtilheiro ‘Woodard’ (Tabela 1).

Tabela 1 – Porcentagem de enraizamento (%), comprimento médio de raiz (cm) e massa seca das raízes (g), de plantas de mirtilheiro 'Woodard' aos 60 dias após o transplante, em função dos tipos e concentrações de auxina.

Pelotas-RS, 2019.

Tipo de auxina	Concentrações (mg.L ⁻¹)			
	0	0,2	0,4	0,6
	Sobrevivência (%)			
AIB	100 ^{NS}	100	100	100
ANA	100	100	100	100
C.V. (%)	0			
	Porcentagem de enraizamento (%)			
AIB	100 ^{NS}	100	100	100
ANA	100	100	100	100
C.V. (%)	0			
	Número de raízes			
AIB	3,52 abA	4,35 aA	3,87 abA	4,10 aA
ANA	3,05 bB	3,47 abB	3,42 abB	3,65 abB
C.V. (%)	10,84			
	Comprimento da maior raiz (cm)			
AIB	4,58 ^{NS} A	4,39 A	4,01 A	4,06 A
ANA	3,90 B	3,83 B	3,54 B	3,57 B
C.V. (%)	20,01			
	Massa de matéria seca da raiz (g)			
AIB	0,18 aA	0,11 abA	0,10 bA	0,10 bA
ANA	0,09 bB	0,09 bB	0,08 bB	0,10 bA
C.V. (%)	30,87			

^{1/} Médias seguidas pela mesma letra minúscula na linha e maiúscula na coluna não diferem entre si pelo teste Tukey ($p \geq 0,05$). C.V: coeficiente de variação. ^{NS}: não significativo.

Pelizza (2009), verificou resultados semelhantes no enraizamento *ex vitro* de mirtilheiro 'Bluebelle' com a utilização de AIB na concentração de 250 mg L⁻¹, obtendo porcentagem de enraizamento de 98,7%.

Segundo Damiani e Schuch (2009), o desenvolvimento de um sistema de enraizamento mais eficiente resulta em mudas com maior qualidade fisiológica e diminuição de perdas durante a fase de aclimatização. Entretanto, pode sofrer interferência de vários fatores, entre eles destacam-se: estado nutricional das plantas, época do ano, taxa de respiração e fotossíntese, bem como a relação carbono/nitrogênio (KRAMER; KOZLOWSKI, 1972).

Para as variáveis número de raízes, comprimento da maior raiz e massa de matéria seca da maior raiz observou-se interação entre os fatores de estudo (Tabela 1).

Quanto ao número de raízes, comparando os tipos de auxina em relação as concentrações, a maior média foi observada no tratamento com o uso de AIB, quando utilizado a concentração $0,2 \text{ mg L}^{-1}$, não diferindo da concentração $0,6 \text{ mg L}^{-1}$.

Os resultados obtidos no presente estudo foram superiores aos observados por Pelizza (2009), que avaliando o enraizamento *ex vitro* de mirtilheiro 'Bluebelle' observou o maior número de raízes (2,97) quando utilizou 250 mg L^{-1} de AIB e substrato vermiculita. De acordo com Sorace et al. (2007), a quantidade de raízes interfere na absorção de água e nutrientes; e consequentemente, pode promover maior desenvolvimento vegetativo.

Em relação ao comprimento da maior raiz, os tratamentos com o uso do AIB propiciaram as maiores médias em relação a ANA. Entre as concentrações, não houve significância estatística para ambas as auxinas (Tabela 1). Segundo Hartman et al. (2002), o uso do AIB estimula as plantas a produzirem raízes maiores e mais fibrosas. Aliado ao uso de auxina, o substrato utilizado no presente trabalho pode ter contribuído para tal resultado.

Martins et al. (2009), descreveram que a vermiculita apresenta vantagens como: fácil obtenção, baixa densidade, uniformidade na composição química e granulométrica, porosidade e capacidade de retenção de água. Em trabalho realizado por Pelizza (2009), avaliando diferentes cultivares de mirtilo, este substrato proporcionou melhores resultados quanto ao comprimento da maior raiz.

Quanto a massa de matéria seca das raízes, ao compararmos os tipos de auxina para cada concentração, o tratamento sem a utilização dos reguladores de crescimento apresentou a maior massa; entretanto, não diferiu dos tratamentos com a utilização de AIB (Tabela 1). Quando comparadas as concentrações, a maior média foi obtida sem o uso do regulador; porém, não diferiu da concentração $0,2 \text{ mg L}^{-1}$ de AIB.

Pelizza (2009), em experimento sobre o enraizamento *ex vitro* de mirtilheiro observou a maior massa quando utilizado 250 mg L^{-1} de AIB e substrato vermiculita para as cultivares Bluebelle e Woodard. De acordo com Tavares et al. (1995), o maior número, comprimento e massa de matéria seca de raízes favorecem o desenvolvimento das plantas e, consequentemente, a qualidade das mudas formadas.

Diante do exposto, o enraizamento *ex vitro* de mirtilheiro 'Woodard' pode ser realizado sem a utilização das auxinas AIB e ANA nas concentrações testadas. Tal fato poderá contribuir na redução no custo de produção das mudas, principalmente em escala comercial, pois além do enraizamento *ex vitro* reduzir o tempo de permanência das mesmas no

laboratório, não será necessário adquirir esses reguladores de crescimento e não haverá demanda de mão de obra para o preparo dos mesmos.

4 CONCLUSÃO

O enraizamento *ex vitro* de mirtileiro 'Woodard' pode ser realizado sem o uso das auxinas ácido indolbutírico e ácido naftaleno-acético.

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Light sources indoor benefit the growth and development of pepper cultivars

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ABSTRACT

Light is a determining factor in plant morphophysiology, as it influences the growth and development of agricultural crops. Thus, the objective was to investigate the effects of light sources on the growth and development of “biquinho” pepper cultivars. The experiment was performed in two stages (Exp1 and Exp2), evaluating two cultivars (BRS Moema; Airetama biquinho) in five light sources (white LEDs, red LEDs, blue LEDs, red/blue LEDs, and fluorescent lamps). In Exp1 the plants were kept in a controlled condition, with a completely randomized design, in a 2x5 factorial scheme (cultivars x light sources) until 76 days after emergence (DAE), in which growth variables and photosynthetic pigments were evaluated. In Exp2, the plants were removed from the above conditions and transplanted in pots, being kept in greenhouse for more 76 DAE. At 152 DAE, the same variables as Exp1 were evaluated, as well as gain of shoot fresh and dry mass, and gain of root fresh and dry mass. In both experiments, growth variables were affected by cultivar and the light sources, however, the behavior did not follow the same trend for all variables, indicating that the light quality influences the growth of the crops, and impacting during greenhouse conditions.

Keywords: *Capsicum chinense*; light quality; light-emitting diodes (LEDs); photosynthetic pigments.

INTRODUCTION

The study of the physiology of plants and their behavior in different environments allowed humans to both select plants and modify their environment to obtain greater productivity and other desired characteristics. However, environmental factors, ie water availability, nutrients and the quality and quantity of light are important for plant growth and development (Guo *et al.*, 2016; Zhang *et al.*, 2019).

Among these environmental factors, light stands out as an important regulator of plant development, morpho-

genesis and physiology, and the quality provided may be able to influence plant productivity. The visible light range, between 400 and 700 nm, is known as photosynthetically active radiation, with the bands of red (600 to 700 nm) and blue (400 to 500 nm) being the ones that most affect photosynthesis, because the molecules of chlorophyll absorb wavelengths in these bands, while the others are usually reflected or transmitted.

Recent innovations in plant growth lighting, particularly through light-emitting diode (LED) technology, allow

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for the creation of customized light spectra, bringing spectra into the decision-making process when a grower or researcher is selecting agricultural lighting (Claypool & Lieth, 2020). LEDs have the flexibility to provide specific wavelengths with the possibility of selecting the emission peak that most closely matches the absorption of photoreceptors, and thus faster and more favorable results for researchers and plant producers (Gupta & Jatothu, 2013; Miler *et al.*, 2019). Using light sources based on LEDs it is possible to regulate and control the physiological aspects of plant growth, such as photosynthesis and/or photomorphogenesis (Gupta & Jatothu, 2013).

In addition, the light emitted from the LEDs is an abiotic elicitor capable of being used in plant morphogenesis, as it has high specificity in wavelengths, allowing researchers to eliminate other wavelengths found in white light, and thus study the stimulating them in the architecture of the plant and in the productive responses. The use of light wavelengths promotes different morphogenetic and photosynthetic responses that may vary among plant species (De Hsie *et al.*, 2019), and in terms of plant production, there are only a few applications where a spectrum of pure monochrome light produces good growth results, such as keeping plants compact or retarding plant growth (Miler *et al.*, 2019).

LED lighting technologies for plant cultivation are rapidly evolving, and lamps for indoor cultivation are often designed to optimize their light emissions in the photosynthetically active spectrum (i.e. red and blue), to reduce energetic requirements for satisfactory yield (Pennisi *et al.*, 2020). This technology has been widely used in horticultural facilities in recent years, however, the influence of light quality should be further explored (Yang *et al.*, 2018). Red and blue lights are currently two types of light spectra that have been most studied on plant photobiology (Zhang *et al.*, 2020).

Plants respond differently to each photosynthetic pigment, the quality of light has varying influences on plant growth and development. The red light (660 nm) and the distant red light (730 nm) affect phytochrome, which affects from seed germination to flowering, fruiting and aging (Oren *et al.*, 2021). The blue-violet light (400-490 nm) regulates plant growth through cryptochromes and phototropins, affecting the growth and development of the root system, and plant stem growth (Pedmale *et al.*,

2016). The yellowish-green light (490-550 nm) is also photosynthetically active radiation (Lin *et al.*, 2020). In *Dendranthema grandiflorum* (chrysanthemum) under red LEDs, increased in height and larger internodes, under blue/red LEDs there was largest fresh weight and greater plant growth, and reduced plant growth kept under blue LEDs (Kim *et al.*, 2004). In *Vaccinium corymbosum* the red light promoted greater rooting and elongation of the stem, and the combinations of blue/red LEDs benefited the accumulation of plant biomass *in vitro* (Hung *et al.*, 2016).

The use of far red inside the canopy of tomato plants resulted in stem elongation, difference in leaf morphology, greater length/width ratio of the leaves, largest leaf area and increase of 7 to 12% in the production of ripe fruits (Zhang *et al.*, 2019). In peppers, the effects of LED lights on growth, yield and fruit quality were tested, with an increase in fruit production, in the dry matter content of the fruits and in the content of fruit-promoting compounds in the fruits (i.e. total phenolic content, carotenoid content and antioxidant activities) (Guo *et al.*, 2016).

In *Capsicum annuum*, plant biomass was reduced when the plants remained under red LEDs in the absence of the blue wavelength, producing characteristics such as greater height and greater mass of the stem, suggesting that the plants require supplementary radiation especially in the blue region for the normal growth and development (Brown *et al.*, 1995). Even with these results, the same authors confirm the physiological and morphological effects of the quality of light, and they can vary according to the species. For cultivars of biquinho pepper (*Capsicum chinense*), light spectra were studied during the seed germination process, concluding that the luminous environment in green, red and far red colors positively influence the germination and the amount of abnormal plants was increased in the dark (Diel *et al.*, 2019). For this species, this is the only report, and it is crucial to study the influence of light source on the morphogenesis and physiology of this culture.

Several studies have revealed the effects of light on the biomass of different species, using LEDs of specific wavelengths. For this, the question remains: which band of the light spectrum can influence the morphogenesis of *Capsicum chinense*? The aim of the study was to evaluate the effects of light sources indoor on the morphogenesis (growth and development) of two cultivars of pepper.

MATERIALS AND METHODS

2.1 Study area and growth conditions

The experiment was carried out at the Federal University of Santa Maria UFSM campus Frederico Westphalen - Rio Grande do Sul, Brazil. The geographical location of this region is 27°22'S, 53°25'O at 480 m of altitude. According to the Köppen classification, the region's climate is Cfa, humid subtropical (Alvares *et al.*, 2013).

For the conduct of the experiment, two steps were performed (Experiment I and II), the first was conducted in a growth room with controlled temperature, humidity and light intensity conditions and the second in a greenhouse.

2.2 Experiment I - Does the light source indoor benefit the vegetative process of biquinho pepper cultivars?

2.2.1 Plant material and propagation

Seeds of two cultivars of "biquinho" pepper, *Capsicum chinense*, (BRS Moema and Aretama Biquinho) were used, characterized by red and yellow fruits when ripe, respectively. The seeds were pre-germinated on Gernitex® paper in transparent plastic Gerbox® and kept in a growth chamber (BOD type) at a constant temperature of 25 °C, 12 hours of light and 12 hours of photoperiod with 36 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance from two fluorescent lamps ("Luz do Dia Especial", 40 W, Osram®, Brazil). To break the seed dormancy, KNO_3 solution (2%) was added, following the standards of MAPA (2009), and after about 15 days after emergence (DAE), when 50% of the seeds showed root protrusion, they were transplanted into transparent plastic cups (300 mL) filled with Carolina® commercial substrate.

From the transplant to plastic cups, the seedlings were kept in a growth room, with a temperature of 25 ± 2 °C, a photoperiod of 16 hours of light and eight hours of darkness, with a luminous intensity of 72 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from different light sources: four fluorescent lamps ("Luz do Dia Especial", 40 W, Osram®, Brazil); two tube lamps (TECNAL TECLAMP®, Piracicaba, Brazil) of different colors [white LEDs, red LEDs (660 nm), blue LEDs (450 nm), red/blue LEDs (660 and 450 nm, with a ratio of 60 and 40%, respectively).

The plants were irrigated manually keeping the humidity constant (to avoid water stress), and the plant nutrition was carried out via fertigation, with nutrient solution (200 g 1000 L⁻¹ of HidrogoodFert®, 250 g 1000 L⁻¹ of calcium

nitrate and 15 g 1000 L⁻¹ of iron EDDHA, Yara®), and electrical conductivity of the solution maintained at 600 μS . The plants remained in growth room conditions until the beginning of flowering, which was determined when 50% of the plants in the plot emitted flowers, that is, with 76 DAE.

2.2.2 Experimental design and variables analyzed

The experimental design used was entirely random in a 2 x 5 factorial scheme, with two cultivars of pepper (BRS Moema and Aretama Biquinho) and five light sources (fluorescent lamps, white LEDs, red LEDs, blue LEDs, and red/blue LEDs), totaling ten treatments with four repetitions per treatment, the experimental unit being composed of four plants, totaling 16 plants per treatment.

At 76 DAE and before transplantation (Experiment II) were evaluated: plant height (PH, cm), number of leaves (NL), shoot fresh mass (SFM, g), shoot dry mass (SDM, g), root fresh mass (RFM, g), root dry mass (RDM, g), root volume (RV, mL), root length (RL, cm), leaf area (LA, m²) and photosynthetic pigments [chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*), total chlorophylls (ChlT), carotenoids (Car) and total chlorophyll/carotenoid ratio (ChlT/Car)].

Before transplanting the plants to the pots, half of them were separated for destructive evaluations of Experiment I and the other half for Experiment II, being evaluated at the end of 152 DAE.

The number of leaves and plant height were evaluated from the emission of the first expanded leaf to the first fork in the main stem of the plant. The root volume was calculated immediately after calculating the fresh mass, from the displacement of water in a graduated cylinder after submersion of the root system. The root length was evaluated by measuring the length of the main root (pivoting root).

The leaf area (LA, cm²) was determined using an LA integrator [model Licor LI- 3100 (LI-COR®, Lincoln, Ne, USA)]. For this, all the leaves were separated from the stem of the plants, being passed one by one in the analyzer.

2.3 Experiment II - Mass gains and quality of plants subjected to light source during the vegetative process of cultivars of biquinho pepper

2.3.1 Plant material

At 76 DAE the plants were removed from the growth room conditions and transplanted in black pots (5 L) filled with typical dystrophic Red Latosol soil, and kept in a

greenhouse with a metallic structure in the shape of an arch, 3.5 m high, 10 and 20 m wide and long, covered with plastic film 150 microns thick, remaining for another 76 days, with a total of 152 DAE (fruit ripening; 50% of plants with ripe fruit).

The plants were irrigated manually, always maintaining constant humidity to avoid water stress. For this purpose, the same fertigation solution mentioned above was used, remaining until 152 DAE.

2.3.2 Experimental design and variables analyzed

This experiment was conducted in a randomized block design in a 2 x 5 factorial scheme, with two cultivars of biquinho pepper (BRS Moema and Airetama Biquinho) and plants from five light sources (cited in Experiment I), totaling ten treatments with ten repetitions per treatment, and each repetition composed of a plant.

At 152 DAE, were evaluated: plant height (PH, cm), number of leaves (NL), shoot fresh mass (SFM, g), shoot dry mass (SDM, g), root fresh mass (RFM, g), root dry mass (RDM, g), root volume (RV, mL), root length (RL, cm), photosynthetic pigments [chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*), total chlorophylls (ChlT), carotenoids (Car) and total chlorophyll/carotenoid ratio (ChlT/Car)] and fresh fruit mass (FFM, g) and dry fruit mass (DFM, g). The mass gain was calculated as a percentage for SFM, SDM, RFM, SDM shoot fresh mass (SFM, g), shoot dry mass (SDM, g), root fresh mass (RFM, g) and shoot dry mass (SDM, g), at 76 DAE and 152 DAE (Experiment I and II). That is, during the period between and greenhouse conditions (76 DAE and 152 DAE, respectively), the masses were related, being calculated as a percentage: gain of shoot fresh mass (%), GSFM), gain of shoot dry mass (%), GSDM), gain of root fresh mass (%), GRFM), gain of root dry mass (%), GRDM).

2.3.3 Photosynthetic pigments

To determine photosynthetic pigments (Experiment I and II), chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids, five disks of 5 mm diameter each were obtained from the second green leaf (from the apex to the base) and incubated in glass test tubes containing 5 mL of dimethylsulfoxide (DMSO) saturated with calcium carbonate (CaCO₃), kept in the dark at room temperature for 48 hours, following the modified methodology of Santos *et al.* (2008), totaling four repetitions per treatment. After 48 hours of darkness, absorbance was determined using

the SPERCORD 50/Plus[®] spectrophotometer (Analytik-jena, Germany) using a 10 mm quartz cuvette. Wellburn (1994) methodology was used to establish wavelength and equations to calculate the concentrations of chlorophylls [*a*, *b*, total (*a* + *b*)] and carotenoids and the unit used was in µg cm⁻².

2.4 Statistical analysis

The variables of both experiments (I and II) were subjected to analysis of variance and the treatment means, when significant, were separated by the Tukey test at 5% probability of error. In addition, based on the desired values of the variables studied, the MGIDI index was used to select the best treatments (Olivoto & Nardino, 2020) and were calculated as follows: $MGIDI_i = [\sum_{j=1}^f (\gamma_{ij} - \gamma_j)^2]^{0.5}$, where MGIDI_i is the multi-trait genotype-ideotype distance index for the *i*th treatment; γ_{ij} is the score of the *i*th treatment in the *j*th factor (*i* = 1; 2; ...; *t*; *j* = 1; 2; ...; *f*), being *t* and *f* the number of treatments and factors, respectively; and γ_j is the *j*th score. Treatments that have the lowest calculated indices are the best. Statistical analyzes were performed with the aid of packages metan (Olivoto & Lúcio, 2020), ggplot2 (Wickham, 2016), ExpDes (Ferreira *et al.*, 2021) available in the R program (R Development Core Team, 2021).

RESULTS

3.1 Experiment I - Light source indoor benefit the vegetative process of biquinho pepper cultivars

From the analysis of variance, there was a significant interaction between cultivars x light sources for the variables PH, NL, SFM, RFM, SDM, RDM and LA. The variables RL and RV were significant only for the cultivars factor, and CR only for light sources. For photosynthetic pigments, there was significant interaction between cultivars x light sources for Chl*a*, Chl*b*, ChlT, Car, ChlT/Car.

The growth variables were affected by both the cultivar and the light sources, in the first stage of the experiment (Figure 1). For the PH variable, the cultivar BRS Moema was superior under blue (15.5 cm) and red LEDs (11.37 cm) but obtained lower averages than Airetama in the fluorescent light source (8.21 cm) (Figure 1A). The same pattern occurred for the variables SFM and SDM (Figure 1C, 1D, respectively). BRS Moema, when kept under LEDs, also obtained higher NL values except for the fluorescent

light source, where NL was higher for the cultivar Airetama (Figure 1B). Differently, RDM presented another behavior when compared to the other variables, with higher averages in the cv. Airetama when grown under Blue LEDs (Figure 1F). For the RFM variable, the inferior performance was observed for the BRS Moema cultivar grown under white

LEDs (Figure 1E).

For the variable RL, the cultivar Airetama had a longer root length when grown under white LEDs (Figure 2A), and shorter when the plants remained under fluorescent and red LEDs (Figure 2C), the same being observed for the variable RV (Figure 2B).

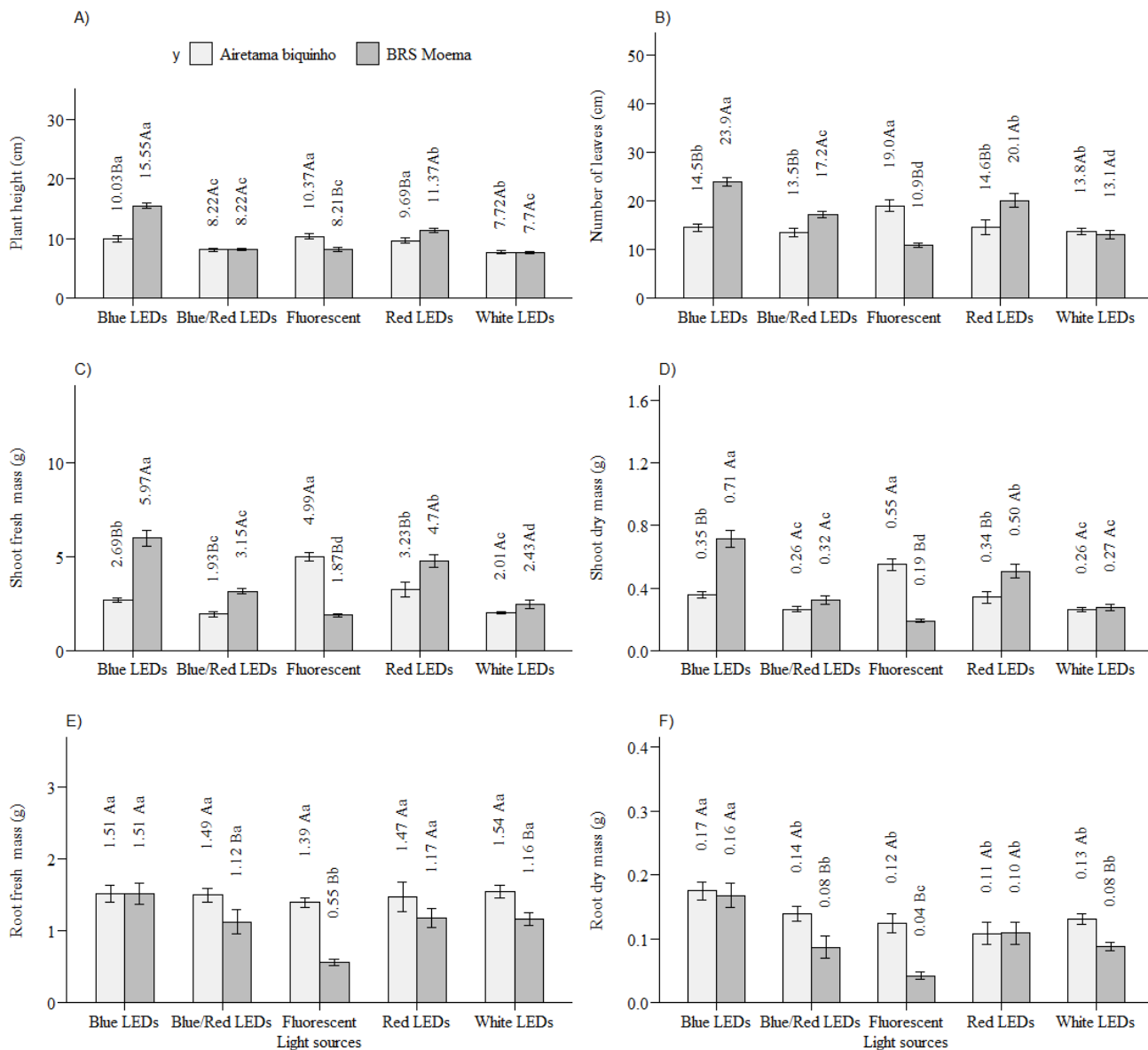


Figure 1: Means of growth variables of biquinho pepper cultivars under different light sources in experiment I: A) plant height (PH, cm), B) number of leaves (NL), C) shoot fresh mass (SFM, g), D) shoot dry mass (RDM, g), E) root fresh mass (SDM, g), F) root dry mass (RDM, g). Lower case letters differ in light sources and upper-case letters differ in cultivars by Tukey's test, at 5% significance.

The cultivar BRS Moema showed superior results of LA when cultivated under blue LEDs and red LEDs. The cultivar Airetama performed better under fluorescent light, while both cultivars showed low performance under the

light sources blue/red LEDs and white LEDs (Figure 3A). For chlorophyll *a* (Chla), the cultivar BRS Moema obtained superior results when cultivated under blue LEDs and blue/red LEDs, and inferior performance under fluorescent light,

in which the cultivar Aretama obtained superior results (Figure 3B). BRS Moema presented superior results of chlorophyll *b* (Chl*b*) under blue LEDs when compared to Aretama, in the other light sources studied, the two cultivars did not present significant differences. The cultivar Aretama did not differ in any of the light sources, while the cultivar BRS Moema obtained a higher Chl*b* content in the blue light source (Figure 3C). Carotenoids showed significant differences between cultivars in the blue/red LED light source, in which BRS Moema was superior to Aretama, and when kept under fluorescent light, the Aretama cultivar was superior to BRS Moema. It was also observed in the light sources in each cultivar, with BRS Moema being superior under blue/red LEDs and Aretama under fluorescent (Figure 3D).

For the variable ChlT, among the cultivars, BRS Moema was significantly superior to Aretama in the blue and blue/red LED light sources, while the cultivar Aretama was superior to BRS Moema in the fluorescent light source, in which it had superior performance when

comparing with all the other light sources studied. BRS Moema had a similar performance when grown under blue, red and blue/red LEDs (Figure 3E). The ChlT/Car ratio showed significant differences between cultivars only under blue LEDs, while within each BRS Moema cultivar it presented a higher ChlT/Car ratio under blue LEDs, and Aretama did not show significant differences between light sources (Figure 3F).

By using the multi-trait index (MGIDI), we selected the 2 best treatments (20%) being T3 (Aretama biquinho and fluorescent light) and T6 (BRS Moema and Blue LEDs), showing the difference in the response of the cultivars in relation to the type of light to which it was submitted (Figure 7A). The two treatments were selected with 90% of the variables with desired values, and only the RV variable did not have the desired selection (SD = -2,35%). Thus, we can say that treatments T3 (Aretama biquinho and fluorescent light) and T6 (BRS Moema and blue LEDs) had a good performance in all analyzed variables, except for RV.

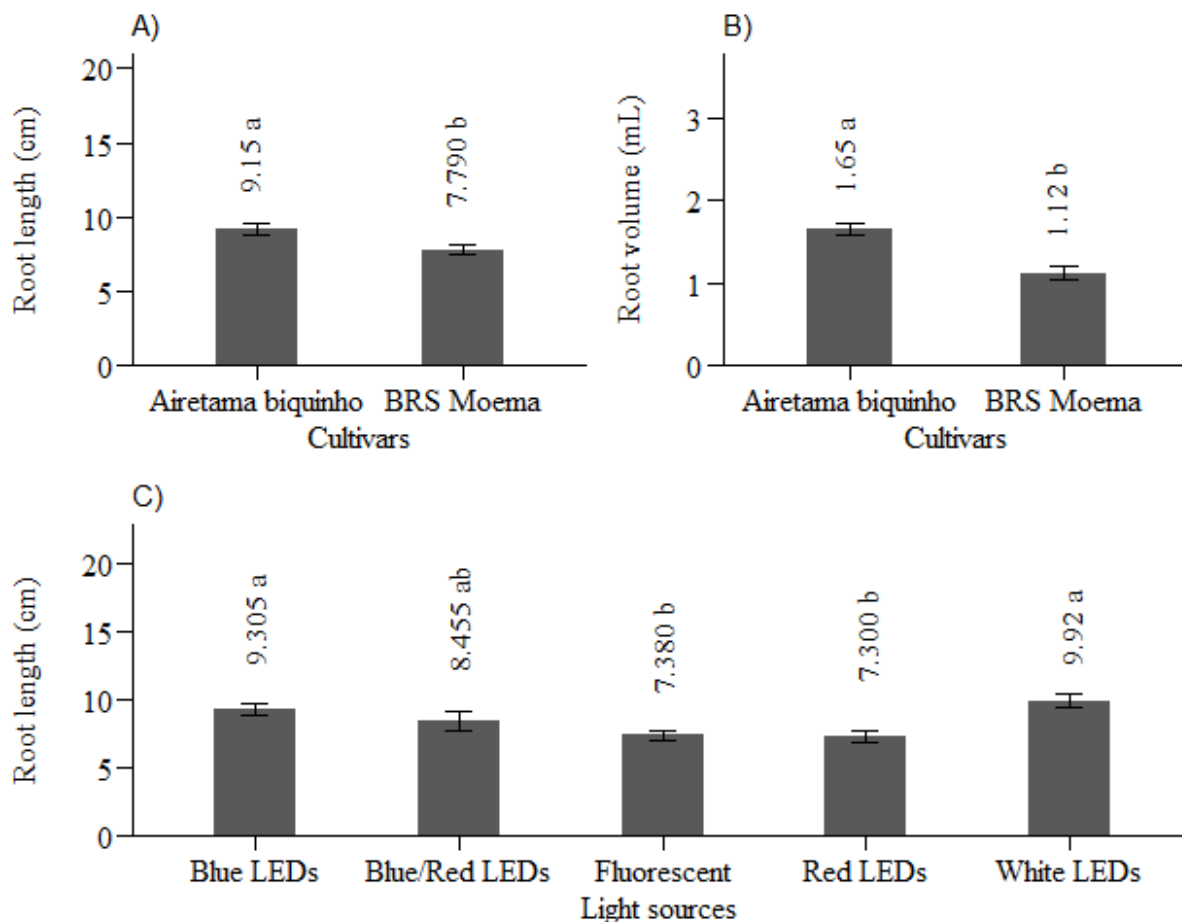


Figure 2: Growth variables (A, C) root length (RL, cm) and (B) root volume (RV, mL) as a function of biquinho pepper cultivars under different light sources in growing room. Lower case letters differ by Tukey's test, at 5% significance.

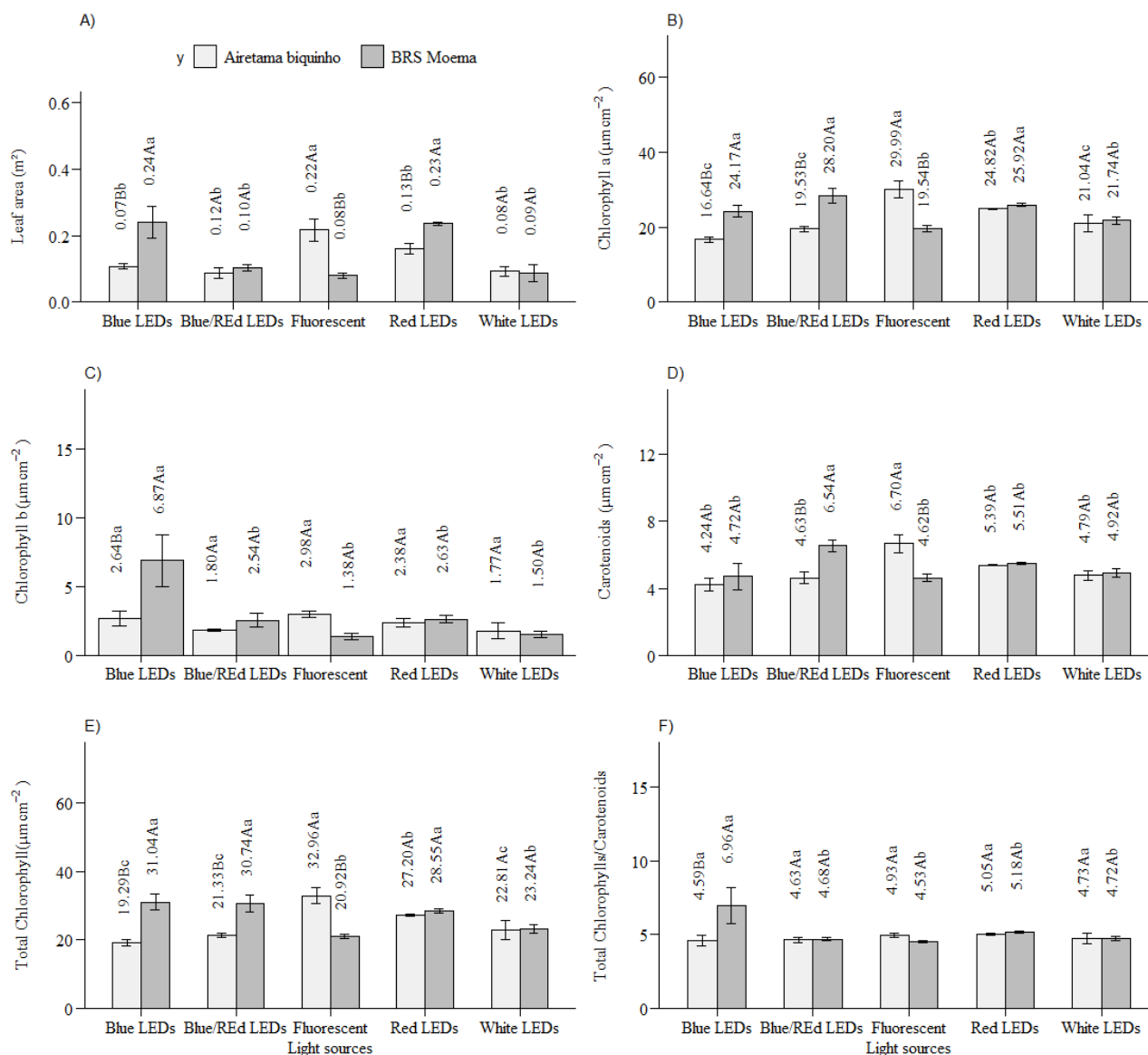


Figure 3: (A) leaf area (LA, m²), (B) chlorophyll *a* (Chl*a*, µm cm⁻²), (C) chlorophyll *b* (Chl*b*, µm cm⁻²), (D) carotenoids (CAR, µm cm⁻²), (E) total chlorophyll content (ChlT, µm cm⁻²), and (F) total chlorophyll/carotenoid ratio (ChlT/Car) of biquinho pepper cultivars grown under different light sources in growing room. Lower case letters differ in light sources and upper-case letters differ in cultivars according to the Tukey's test, at 5% significance.

3.2 Experiment II - Mass gains and quality of plants subjected to light sources during the vegetative process of cultivars of biquinho pepper

From the analysis of variance, it was possible to observe that there was interaction between cultivars x light sources for the growth variables RV, SFM, RSM, RDM. For PH, NL, SDM and RL there was no significant difference. For photosynthetic pigments, there was interaction between cultivars x light sources for the variables Chl*a*, ChlT and Car, whereas for Chl*b* and ChlT/Car ratio there was no

significant effect. For the variables of mass gain (GSFM, GSDM, GRFM, GRDM) there was a significant interaction between cultivar x light sources.

The variable RV, after transplanting the seedlings to pots kept inside the greenhouse, the behavior of the cultivar Aretama was superior to BRS Moema when grown under blue LEDs and red LEDs. In the other light sources, the cultivars did not differ significantly. For each cultivar, Aretama showed superior results when kept under blue LEDs and red LEDs, and the BRS Moema cultivar showed no significant differences for light sources (Figure 4A).

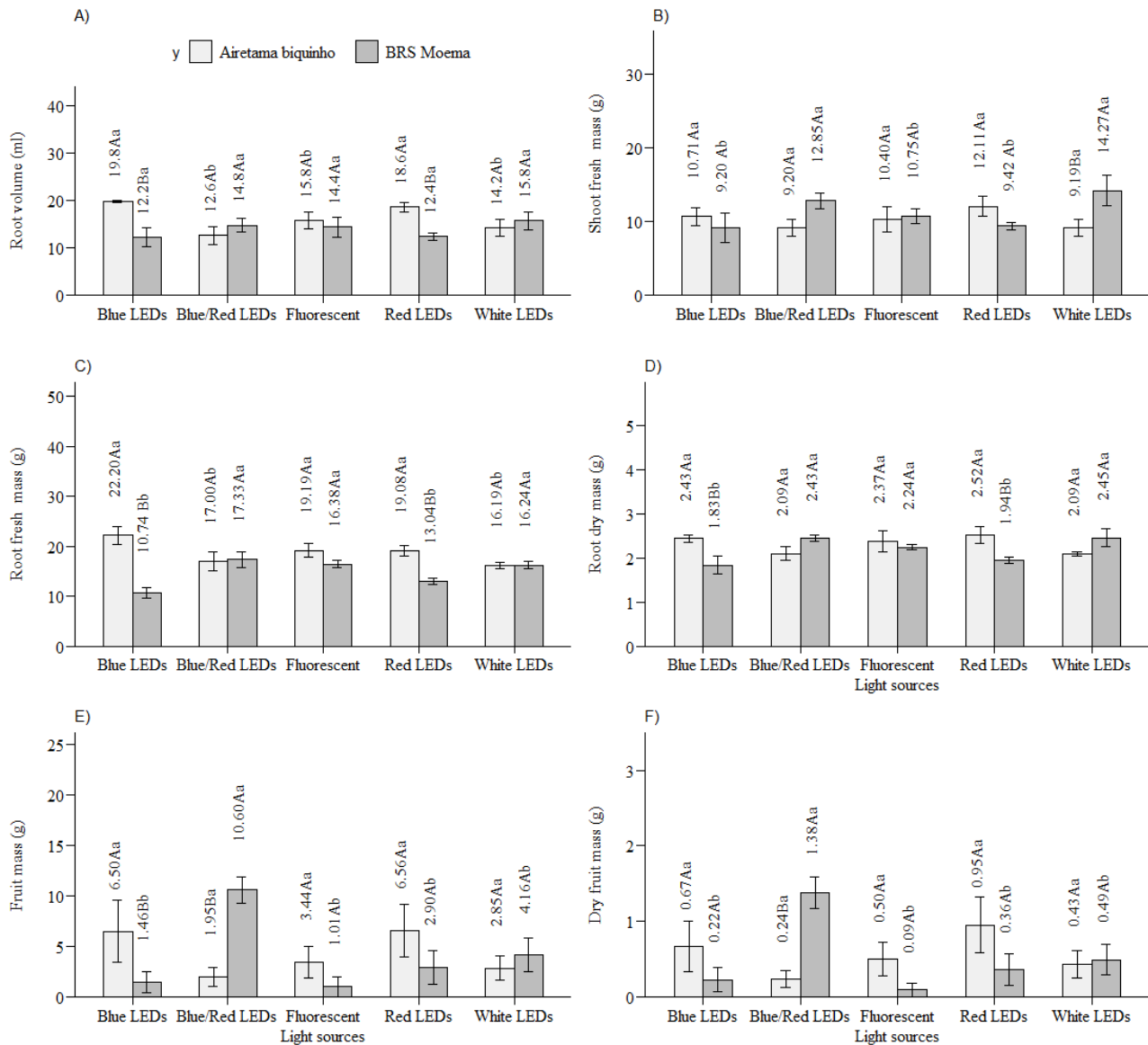


Figure 4: (A) root volume (RV, ml), (B) shoot fresh mass (SFM, g), (C) root fresh mass (g), (RFM, D) root dry mass (RDM, g), E) fresh fruit mass (FFM, g) and F) dry fruit mass (DFM, g) of piquet pepper cultivars at 152 DAE in greenhouse. Lower case letters differ in light sources and upper-case letters differ in cultivars according to the Tukey test, at 5% significance.

As for photosynthetic pigments, contrary to what was observed in Experiment I, lower levels of Chl a were observed for the BRS Moema cultivar when comparing Aretama under blue LEDs, however the fluorescent light continued to be the one with the lowest Chl a values of the BRS Moema cultivar (Figure 5A). For Chl T , among cultivars, different from experiment I, Aretama was significantly superior to BRS Moema in blue LEDs and under fluorescent lamps, while the cultivar BRS Moema was significantly superior to Aretama when kept under

red LEDs. Within each cultivar, BRS Moema showed higher Chl T content when the plants were under red and blue/red LEDs. Under blue LEDs, Aretama showed superior performance (Figure 5B). There were significant differences between cultivars for carotenoids, that is, the cultivar Aretama when grown under blue LEDs and fluorescent lamps was superior to BRS Moema, while the latter was significantly superior under red LEDs. Within each cultivar, this variable followed the same trend found for the Chl T variable (Figure 5C). Analyzing the results

obtained, we can infer that each cultivar has different characteristics regarding the production of photosynthetic pigments and in relation to each light source.

The mass gain was significant for all treatments (light sources x cultivar), for 76 days passed from the initial to the final evaluation. For GSFM, the lowest mass gain of the cultivar Airetama was observed in the fluorescent light source (101.4%), while for the cultivar BRS Moema the lowest mass gains were observed under blue LEDs (57.3%) (Figure 6A). For GSFM, the cultivar Airetama had no differences between the light sources, while for the cultivar BRS Moema higher values were observed when they were under fluorescent and white LEDs (1338.3 and 1240.1% respectively). Another important factor was observed for the fluorescent source, which showed low GSDM for the cultivar Airetama (332.6%), while for BRS Moema it was superior (Figure 6B).

For GRFM, the cultivar Airetama did not differ between the light sources evaluated, while for the cultivar BRS Moema, when they were subjected to fluorescent sources, it presented the greatest gains compared to LEDs (2578.3%). In comparison to cultivars within each light source, the differences were also related to gains in cultivation under fluorescent (Figure 6C). The same trend was observed for the GRDM variable, in which the greatest gains were achieved under fluorescent lamps for the cultivar BRS Moema (5213.6%) (Figure 6D).

Regarding the multi-trait index (MGIDI), as in experiment I, the 2 best treatments were also selected (20%) being T1 (Airetama biquinho and blue LEDs) and T3 (Airetama biquinho and fluorescent light), showing the difference in the response of the light used (Figure 7B). The two treatments were selected with 60% of the variables with desired values, and the variables RL, NL, SFM and SDM did not have the desired selection ($SD = -5.3\%$, -9.57% , -2.37% , and -0.56% , respectively). Thus, we can say that treatments T1 (Airetama biquinho and blue LEDs) and T3 (Airetama biquinho and fluorescent light) had a good performance in most of the variables analyzed.

DISCUSSION

This is the first report on the use of light source in the cultivation of *Capsicum chinense* cultivars kept under con-

trolled conditions (Experiment I) and the impact of these on the growth and development of plants in greenhouse cultivation (Experiment II) to infer about the increment and gain of the plants in those conditions.

For most of the variables analyzed, there was an interaction between cultivars x light sources evaluated in the study. It is known that pepper genotypes can respond differently to environmental stimuli, and depending on the environment, they can produce, for example, different amounts of capsaicinoids and have differences in development (Jeeatid *et al.*, 2018).

The cultivars showed different responses to the light sources in which they were submitted. For example, the plant height (PH) of the cultivar BRS Moema was higher when they were associated with blue LEDs and red LEDs. The spectrum of blue and red light also impacted the height of petunia flowers, due to the elongation of the stem, due to the blue light stimulating the production of gibberellins, and thus causing changes in height (Fukuda *et al.*, 2016). However, the wavelengths in blue and red can positively or negatively affect the growth and development of the species, and this is because these wavelengths are responsible for the activation of different genetic expressions in plants (Jeeatid *et al.*, 2018).

For growth variables, plants from cultivar BRS Moema showed superior results when grown under monochromatics blue or red LEDs. In contrast, the cultivar Airetama showed greater growth when cultivated under a source of fluorescent light and white LEDs; this reveals that the responses between genotypes of the same species may be different (Jeeatid *et al.*, 2018), and also demonstrated by MGIDI in Experiment I. Fluorescent lamps commonly applied, generally lack a wavelength in red, which is very important for the development of plants, for example in stem elongation and phytochrome activity, while these lamps emit a lot in the green and yellow light spectra, which are less efficient for plants (Miler *et al.*, 2019). In *Capsicum annuum*, a broad spectrum including red, green, and blue wavelengths resulted in the highest shoot dry weight and plant compactness, and under monochromatic blue LEDs, these authors observed a reduced leaf area under blue LEDs (Claypool & Lieth, 2020); differently from the present work, suggesting that this may be a species-specific response.

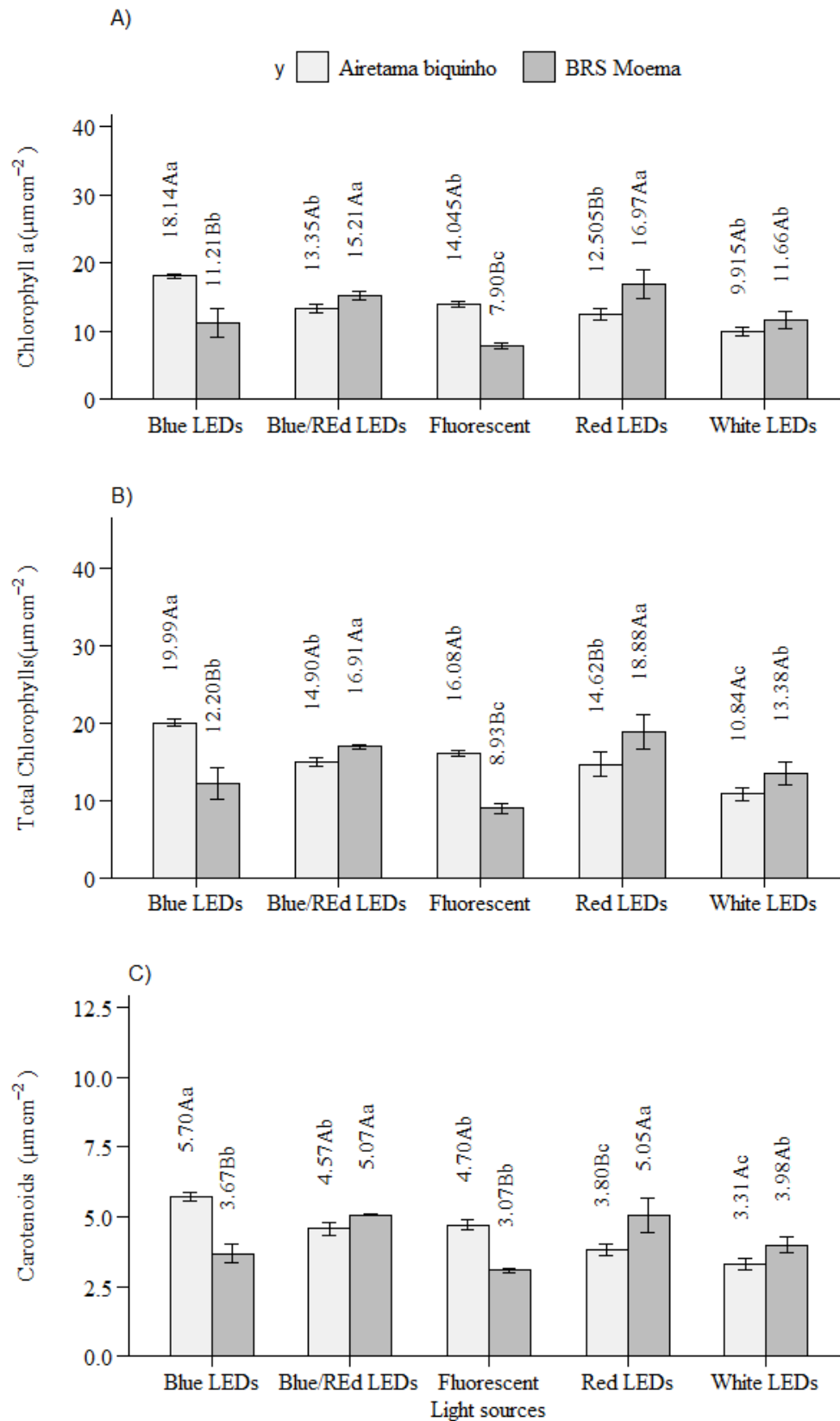


Figure 5: (A) chlorophyll *a* (Chl*a*, $\mu\text{m cm}^{-2}$), B) total chlorophyll (ChlT, $\mu\text{m cm}^{-2}$), C) carotenoids (Car, $\mu\text{m cm}^{-2}$) from biquinho pepper cultivars grown at 152 DAE in greenhouse. Lower case letters differ in light sources and upper-case letters differ in cultivars according to the Tukey's test, at 5% significance.

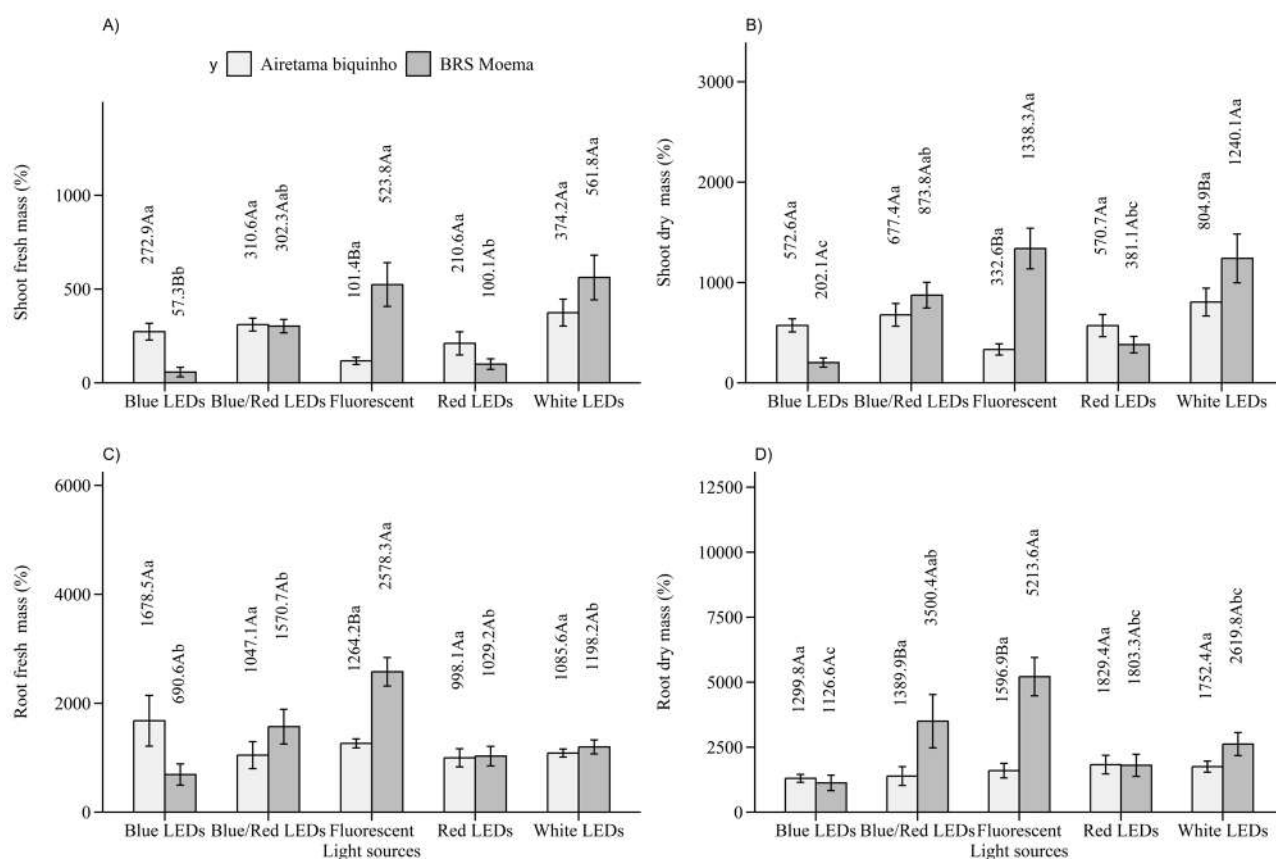


Figure 6: Mass gain as a percentage of 76 DAE to 152 DAE for (A) gain of shoot fresh mass (GSFM, %), (B) gain of shoot dry mass (GSDM, %), (C) gain of root fresh mass (GRFM, %) and (D) gain of root dry mass (GRDM, %) for Airtama and BRS Moema cultivars at 152 DAE in greenhouse. Lower case letters differ in light sources and upper-case letters differ in cultivars according to the Tukey's test, at 5% significance.

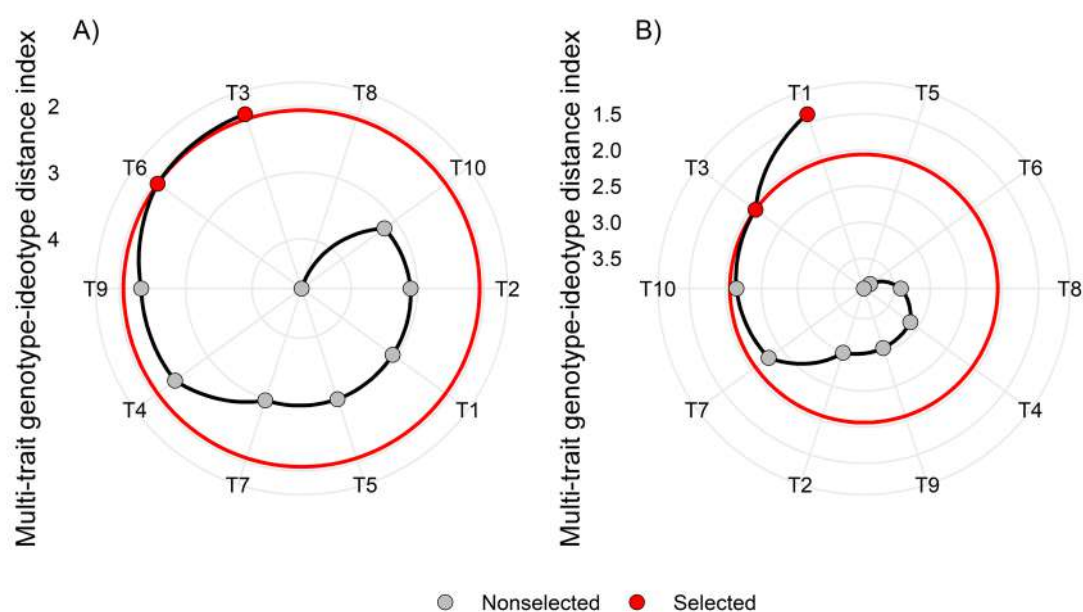


Figure 7: Treatments ranking in ascending order for the MGIDI index. The selected treatments are shown in red color and the red circle represents the cutpoint according to the selection pressure. T1: Airtama biquinho and Blue LEDs, T3: Airtama biquinho and fluorescent light and T6: BRS Moema and Blue LEDs.

Chlorophylls are photosynthetic pigments that absorb light to provide photosynthetic energy to plants (Fang *et al.*, 2022) and contribute to plant vigor due to photosynthetic efficiency (Huang *et al.*, 2013), mainly comprise chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Car). Chlorophyll (Chl) absorb energy from sunlight and convert the light into chemical energy (Björn, 2015), while carotenoids have the function of collecting light in the range of 450-570 nm and protecting plants against photooxidative damage (Frank & Cogdell, 1996). For photosynthetic pigments, the cultivars showed different responses, while the contents of Chla, Chlb and carotenoids in the cultivar BRS Moema were higher results for the blue, red LEDs and their combination. Shin *et al.* (2008), also observed that pigment biosynthesis was increased in *Doritaenopsis* plants grown with the plus of red and blue LEDs. These same authors report that the use of this mixed red and blue radiation can benefit the quality production of plants. The cultivar Airetama showed higher values of Chla and Chlb for the fluorescent light, in contrast on lettuce (Chen *et al.*, 2014) and *Doritaenopsis* (Shin *et al.*, 2008) both under fluorescent lamps. It is therefore difficult to indicate a single spectrum of light suitable for the propagation of all plant species (Miler *et al.*, 2019), and in the present work, this response was species-specific in many of the analyzed variables.

There was a change in the behavior of BRS Moema plants at 76 DAE, when they were compared to 152 DAE for the variables SFM, RFM e SDM. At 76 DAE, these plants showed superiority when grown under blue LEDs, however, at 152 DAE, this result changed, with those grown under fluorescent and white LEDs with greater increment; suggesting that plant growth responses to light quality may not only be associated with species or cultivars, but also the plant growth stages (Chen *et al.*, 2014).

The use of artificial lighting has a positive effect on the growth and development of the biquinho pepper cultivars, proven by the gain of differential biomass between them. The greatest gains of SFM and SDM for BRS Moema cultivar were under fluorescent and white LEDs, for the variables RFM and RDM the greatest gains were observed in the fluorescent. The cultivar Airetama showed no differences ($p > 0.05$) for mass gain in different light sources.

The increase gains attributed to the fluorescent and white LEDs in the period; may be related to the phase in which the plant is at the moment (Chen *et al.*, 2014). In contrast, Ajdanian *et al.* (2019), observed superiority with the application of blue and red light, as there was an increase in fresh and dry masses of cress (*Lepidium sativum*), as well as their biomass compared to plants grown under natural sunlight conditions.

Due to the large number of treatments tested (2 cultivars and 5 light sources = 10 treatments) it can be difficult to select the treatments that present the greatest selection differential, that is, desired characteristics. Using MGIDI we have the possibility to find the best treatments in a simple way and based on a multitrait framework (Olivoto & Nardino, 2020).

Light is an important signal element that regulates plant morphology, physiology and development during the plant growth cycle (Chen *et al.*, 2014), and the application of different light sources and their effects on plants is positive, as they result in the improvement of the biochemical characteristics and stimulates the use of these light sources in an effort to increase the quality of the plant and understand biochemically what happens with this application, and how it influences plant morphology. And this is only possible due to advances in technology, in which they must be constantly updated, and simultaneously stimulates the use of these in new studies and applications by researchers worldwide, and can also be used as allies in future plant production.

CONCLUSIONS

The results of the present study indicate that the light sources emitted in part of the growth of the plants becomes crucial during greenhouse conditions when these plants were transferred to soil. The treatments selected under greenhouse conditions are T3 (Airetama biquinho and fluorescent light) and T6 (BRS Moema and Blue LEDs) and in field conditions T1(Airetama biquinho and Blue LEDs) and T3 (Airetama biquinho and fluorescent light). Our discovery achieved the proposed objective, demonstrating that the use of LEDs is effective in the development and more vigorous growth of *Capsicum chinense*, and that this response is dependent cultivar.

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CONFLICT OF INTERESTS

There is no conflict of interests in carrying this research and publishing this manuscript.

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CROP SCIENCE

Light quality and sealing type affect *in vitro* growth and development of *Capsicum frutescens* cultivars

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Abstract: The aim of this work was to evaluate the effect of five light qualities [fluorescent lamps (FL); white (WL), red (RL), blue (BL), or red/blue LEDs (RBL)] and two types of flask sealing (with or without porous membranes) in the *in vitro* germination and growth of two *Capsicum frutescens* cultivars (Espagueteinho and Cayenne). For this, two seeds were inoculated per flask (350 mL) containing 50 mL of MS medium with 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose, 6.0 g L⁻¹ agar, and pH 5.8 ± 0.1. The plants were kept under culture room conditions (16-h photoperiod, 25 ± 2°C and irradiance of 40 μmol m⁻² s⁻¹). The experiment was arranged in CRD, 2x5x2 factorial scheme (Cultivar X Light qualities X Sealing type). At 42 days, the following traits were evaluated: percentage of germination; root and shoot length; number of green leaves, senescent leaves and nodal segments; and fresh and dry weight. Plants grown under RL and BRL, showed greater number of green leaves, and longer shoots and roots. The use of porous membranes remarkably reduced the number of senescent leaves. Thus, the use of porous membranes, RL and BRL are recommended since they improve growth and development of pepper *in vitro*.

Key words: Pepper, Light-emitting diodes (LEDs), porous membranes, gas exchange.

INTRODUCTION

Pepper (*Capsicum* sp.) is a horticultural crop cultivated in tropical and temperate regions. Besides its high ornamental and medicinal value, its fruits are used in jellies, in the manufacture of preserved foods, sauces, spices or powdered for seasoning (Sanatombi & Sharma 2007, Valnir Júnior et al. 2015, Swamy et al. 2017). Among the uses of these plants, the medicinal application due to the high contents of capsaicin alkaloid in the fruits, which confers the pepper pungency of dihydrocapsaicin, homodihydrocapsaicin and homocapsaicin, all known as capsaicinoids (Davis et al. 2007, Mueller-Seitz et al. 2008). The capsaicinoids are synthesized in the fruits

placenta and some factors such as temperature, nutrient availability, nitrate fertilization and carbon availability may affect the accumulation of these substances (Garruña-Hernandez et al. 2013, Aguilar 2016).

Usually, the propagation of *Capsicum* plants is performed sexually. However, this seed-based propagation method can be limited by several pathogens, such as fungi, nematodes and viruses. So, *in vitro* propagation stands out as an alternative to allow a rapid production and multiplication of this genus, generating uniform, large-scale and disease-free plant material (Sanatombi & Sharma 2007, Chandra et al. 2010). Considering the susceptibility of peppers to diseases and pests, the improvement of tissue

culture techniques is very important. However, some authors report recalcitrance of the genus *Capsicum* for *in vitro* regeneration, which may hinder the applicability of genetic improvement techniques and molecular biology (Kothari et al. 2010, Mohamed & Alsadon 2011, Batista et al. 2017, Gammoudi et al. 2018). In addition, some authors report that this recalcitrance has a positive correlation to ethylene levels produced during *in vitro* growth and development, making it a limiting factor for species such as pepper (Santana-Buzzy et al. 2006, Batista et al. 2013, Gammoudi et al. 2018). Also, among the factors required for the success of *in vitro* regeneration, are the genotype, the light regime and the effect of gas exchange (Dabauza & Peña 2001, Ochoa-Alejo & Ramirez-Malagon 2001, Mohamed & Alsadon 2011, Batista et al. 2017). Especially in peppers of the genus *Capsicum*, the physiological responses are extremely genotype-dependent, which makes necessary studies directed to the cultivars of interest (Dabauza & Peña 2001, Steinitz et al. 2003).

In customary *in vitro* plant cultivation tight sealed flasks have been widely used. Remarkably, there is a restricted gas exchange leading to accumulation of ethylene and other gases, reduced gas exchange, low CO₂ concentration, high carbohydrate, low photosynthetic and transpiratory rates of the plants, lessening their rate of multiplication and survival during acclimatization (Aragón et al. 2010, Kozai 2010, Xiao et al. 2011, Nguyen et al. 2016, Batista et al. 2018). Notably, to overcome such constraints and improve the *in vitro* environment, porous membranes have been used. They promote gas exchanges resulting in increased inner CO₂ concentrations, reduced humidity and ethylene concentrations, stimulating photosynthesis and benefiting explants growth and allowing the photoautotrophic propagation (Kozai & Kubota 2001, Saldanha et al. 2012). Ultimately,

it favors to rustify *in vitro* grown plants during acclimatization, which is a delicate step for tissue culture.

The development and biological processes of the plants are influenced by light spectrum stimuli, interfering in the size and number of organs, number of shoots, seed germination, determination of growth and flowering habits, fruiting, among others (Morelli & Ruberti 2000, Carvalho et al. 2011). Under *in vitro* conditions, light stands out as an important factor, supporting plant growth and development (Muleo et al. 2001, Pawłowska et al. 2018). Although these plants depend on the sugars present in the culture medium as energy source, light is still an important regulator of plants development (Pawłowska et al. 2018). Advances in light technology led the researchers to explore light-emitting diodes (LEDs) in tissue culture, demonstrating great potential for their use in *in vitro* cultivation due to long lifespan, low power consumption, small mass and volume, low radiant heat emission, high wavelength specificity and capacity of emitting specific spectral qualities (Hung et al. 2016, Batista et al. 2018, Pawłowska et al. 2018).

The wavelengths of red and blue light are the focus of current research due to their regulatory effects on plant development, since they promote photosynthesis (Bantis et al. 2016). The red light (660 nm) is efficiently absorbed by photosynthetic pigments (chlorophyll and carotenoids) and stimulates the phytochrome fotoreceptor. The blue light (400-500 nm), by its turn, performs a variety of important photomorphogenic roles in plants, including stomatal opening, stem elongation and phototropism (Pawłowska et al. 2018).

Therefore, this study hypothesis that different light qualities and sealing types modify the *in vitro* germination and growth of *Capsicum frutescens* cultivars.

MATERIALS AND METHODS

Plant material and growing conditions

The experiment was carried out at the Tissue Culture and Aromatic Extracts Laboratory of the Federal University of Santa Maria, Frederico Westphalen, RS, Brazil.

Seeds from two cultivars of *Capsicum frutescens* namely 'Espaguetinho' and 'Cayenne' (ISLA®) were disinfested in 70% alcohol for 1 min, followed by sodium hypochlorite (NaClO) at 0.8% active chlorine for 15 min, and four rinses in autoclaved distilled water. After disinfestation, two seeds were inoculated into each glass flask (350 mL capacity) containing 50 mL of macronutrients, micronutrients and vitamins of the MS culture medium (Murashige & Skoog 1962), with 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose, and 6.0 g L⁻¹ granulated agar. The pH was adjusted to 5.8 ± 0.1, prior to autoclaving at 120 °C, 108 kPa, for 15 min. The cultures were kept under culture room conditions with a 16-h photoperiod, temperature of 25 ± 2 °C and irradiance of 72 µmol m⁻² s⁻¹ from five different light qualities: four fluorescent lamps (FL, Luz do Dia Especial, 40 W, Osram, Brazil); two white LEDs (WL), red LEDs (RL), blue LEDs (BL) or red/blue LEDs (RBL, 60 and 40%, respectively), TEC LAMP®. In addition, two types of sealing were used: rigid metal lids without orifice and rigid metal lids with two 10 mm-diameter holes covered by porous membranes, which were prepared following the methodology proposed by Saldanha et al. (2012).

Analyzed characters

After 42 days of *in vitro* cultivation the following characters were evaluated: percentage of germination, root and shoot length, number of green leaves, number of nodes, fresh and dry weight, number of senescent leaves and

photosynthetic pigments (chlorophyll *a*, *b* and carotenoids).

Quantification of photosynthetic pigments

Three 3 mm-diameter leaf discs were removed from the second and third leaf of the apex towards the base and incubated in 3 mL of saturated dimethylsulfoxide (DMSO) with CaCO₃, following the modified methodology proposed by Santos et al. (2008). The discs remained in glass tubes covered with aluminum and kept in the dark for 48 hours at room temperature. After this, the absorbance of the samples was determined using a 10 mm quartz cuvette of optical path in Bel Photonics® SP 1105 spectrophotometer. The wavelengths and the equations to calculate chlorophylls *a*, *b*, and carotenoids concentrations were based on the methodology described by Wellburn (1994).

Experimental design and statistical analysis

The experiment was arranged in a completely randomized design (CRD), in a 2x5x2 factorial scheme (two cultivars, five light qualities and two types of sealing), totaling 20 treatments, with 10 replicates each. The experimental unit consisted of two plants per flask.

For the photosynthetic pigments analyzes three replicates were used, each one composed of a test tube containing three leaf discs. The data was submitted to Shapiro-Wilk's test and the growth parameters (root length, shoot length, number of green leaves, number of nodal segments, fresh weight, dry weight and number of senescent leaves) were transformed to $y = \text{square root of } x + 0.5$, to meet the normality assumptions (data is presented with non-transformed means). Data were submitted to analysis of variance by F-test and regression analysis for the quantitative data (days after inoculation). For qualitative data, the means were compared by the Tukey's test at 5%

significance level. All statistical analyzes were performed using the statistical program SISVAR (Ferreira 2011).

RESULTS

By the results of analysis of variance, root length, fresh weight and dry weight differed for the triple interaction among cultivars x light qualities x types of sealings. For the number of green leaves and number of nodal segments, there was a significant difference for cultivars x types of sealings and for light qualities. For number of senescent leaves, there was a significant difference for light qualities and types of sealing. For shoot length, there was a significant difference only for the light qualities. For percentage of germination, there was difference only for light qualities x types of sealing and for days after inoculation for both cultivars ('Espaguetinho' and 'Cayenne') ($p < 0.05$, data not shown).

Growth characters

For percentage of germination in the 'Espaguetinho', it was possible to infer that the red LED with membrane was significantly higher than other light qualities, and no difference was observed in the type of sealing without porous membranes (Figure 1h; Figure 2a). For the 'Cayenne', there was a significant difference only for red/blue LEDs without porous membranes, where the highest percentage of germination was observed (Figure 2b; Figure 3e).

The red and red/blue LEDs were showed more senescent leaves compared to the blue LED (Figure 4a), and plants produced under these blue light conditions generated reduced amount of green leaves (Figure 4c). Also, plants grown without membrane have more senescent leaves than those cultivated in flasks with

membranes (Figure 4b). The number of green leaves formed under red LEDs and red/blue LEDs was significantly higher as compared to white and blue LEDs and fluorescent lamps (Figure 4c). For cultivar x types of sealing, the 'Espaguetinho' without membrane showed more leaves than with membrane, while the 'Cayenne'

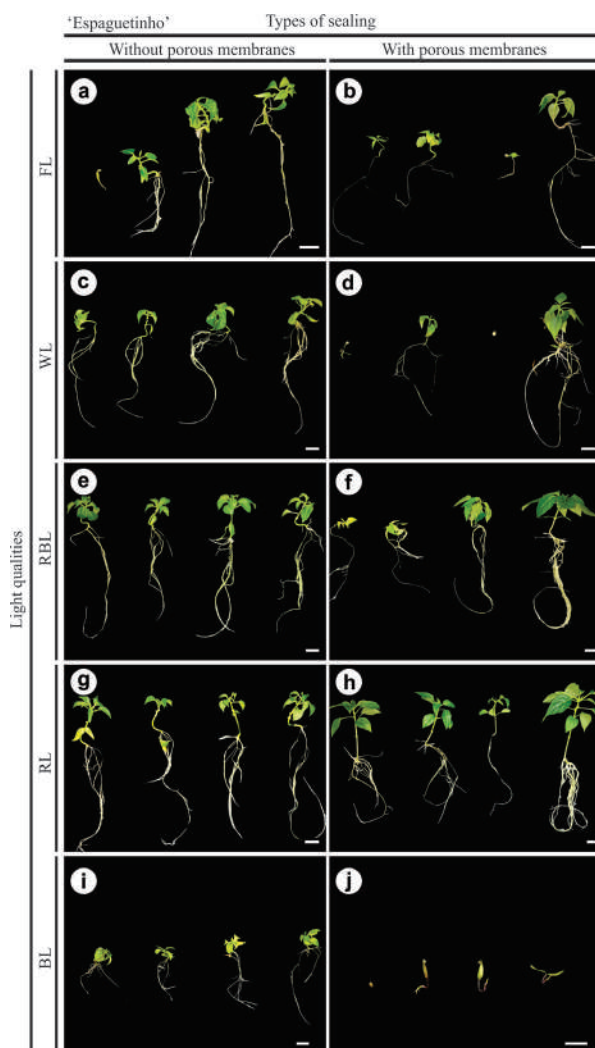


Figure 1. Plants of *Capsicum frutescens* cv. 'Espaguetinho' cultivated *in vitro* under different light qualities and types of sealing: Fluorescent (FL) without (a) and with porous membranes (b); White LED (WL) without (c) and with porous membranes (d); Red/blue LED (RBL) without (e) and with porous membranes (f); Blue LED (BL) without (g) and with porous membranes (h); Red LED (RL) without (i) and with porous membranes (j). Bars: 1 cm.

did not differ between the types of sealing. (Figure 4d).

The plants cultured in red/blue LEDs showed greater number of nodal segments, despite did not differ statistically from white and red LEDs (Figure 5a). For cultivar x types of sealing, plants grown in flasks without membrane had more nodal segments than those with membrane for 'Espagueteinho' and for 'Cayenne', the types of sealing were similar for this feature. 'Cayenne' had more nodal segments than 'Espagueteinho' in the treatments with membrane while without membrane there was no difference between the cultivars (Figure 5b).

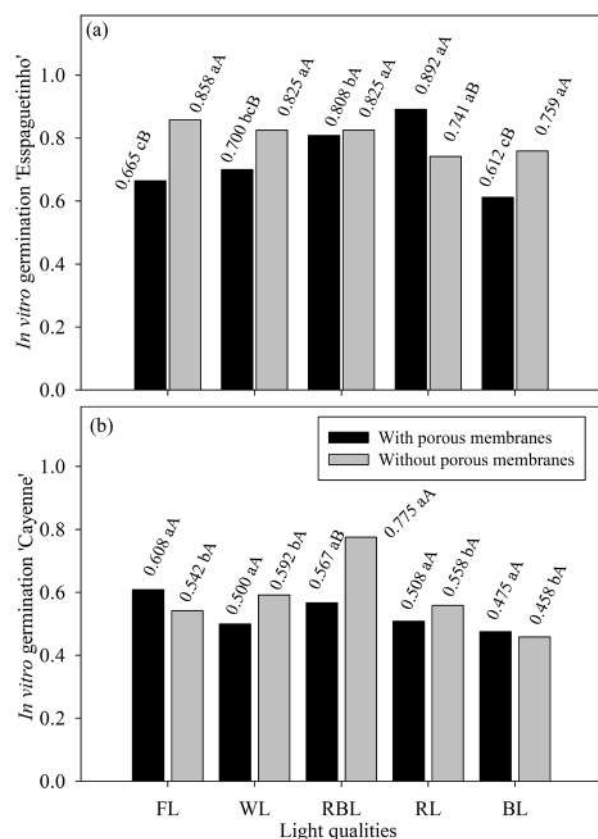


Figure 2. *In vitro* germination of *Capsicum frutescens* cv. Espagueteinho (a) and Cayenne (b), evaluated after 42 days of culture under different light qualities and types of sealing. *Lowercase letters in type of sealing (with or without membrane), and uppercase letters in the same light quality do not differ by the Tukey's test at 5% level.

Plants cultured in red LEDs developed longer shoots, followed by those grown under red/blue LEDs (Figures 1; 3; 5c). For root length, there was a triple interaction among the factors. Considering cultivar x light qualities x types of sealing between the 'Espagueteinho' and 'Cayenne' in the white LED, the former without membrane differed statistically from the one with membrane, with greater root length, whereas the latter did not differ. 'Espagueteinho' under blue LEDs and without membrane was superior from the one with membrane, while to 'Cayenne' they did not differ (Table I).

'Espagueteinho' plants grown under red LEDs in with membranes developed longer roots than those cultured in other light qualities. For the treatments without membrane, there was no significant difference between the light qualities instead. For 'Cayenne' with membrane, the lights fluorescent, and white, red/blue and red LEDs had longer roots than those grown under blue LEDs, the same occurred for the treatment without membrane in this cultivar. The comparisons among the lights red/blue, red and blue LEDs did not varied in root length between the cultivars, just as for the types of sealing in red/blue, red and blue LEDs (Table I; Figures 1e, 1f, 1g, 1h, 1i, 1j and 3e, 3f, 3g, 3h, 3i, 3j).

For fresh weight there was also a triple interaction among the treatments and both cultivars did not differ when analyzed in the fluorescent lamp and blue LED. However, 'Espagueteinho' without membrane differed from the one with membrane with greater fresh weight, while 'Cayenne' did not differ. For both cultivars, the treatment without membrane was superior from the one with membrane in the red/blue LED. In the red LED, 'Espagueteinho' did not present any significant difference. Unlike, for 'Cayenne' the treatment without membrane produced plants with greater fresh weight (Table II).

For light qualities x cultivar x types of sealing, the ‘Espagueteinho’ under the red LED was superior only from the white and blue LED in the type of sealing with membrane, while in the treatment without membrane, the red/blue LED was superior only from the fluorescent lamp and blue LED. Within ‘Cayenne’ with membrane, the light qualities did not show statistical

difference among the treatments; and for the treatment without membrane, the red/blue LED produced plants with greater fresh weight than all the other light qualities (Table II). When compared to membrane treatment between the two cultivars, there was no significant difference among the treatments in the light qualities. For the treatment without membrane, ‘Cayenne’ was superior from ‘Espagueteinho’ in the red/blue LED and red LED, while the other treatments did not differ among themselves (Table II).

For dry weight there was also a triple interaction. Thus, considering cultivar x light qualities x types of sealing, ‘Espagueteinho’ did not differ statistically, and the ‘Cayenne’ without membrane was superior when compared to the one with membrane with red/blue and red LEDs, while the other treatments did not differ for this cultivar. For light qualities x cultivar x types of sealing in the ‘Espagueteinho’ there was no difference among the light qualities; the ‘Cayenne’ in the treatment without membrane and red/blue LED produced plant with dry weight greater than all the other light qualities; while in the treatment with membrane for this cultivar there was no difference among the light qualities (Table III).

Considering the types of sealing x cultivar x light qualities, only the treatment with membrane and red LEDs differed statistically between the cultivars, showing higher values for dry weight, while in the treatment without membrane in the white LED, and ‘Espagueteinho’ was superior; in red/blue LED for ‘Cayenne’ the treatment without membrane was higher. For the other treatments in the cultivar Cayenne, the types of sealing did not differ statistically (Table III).

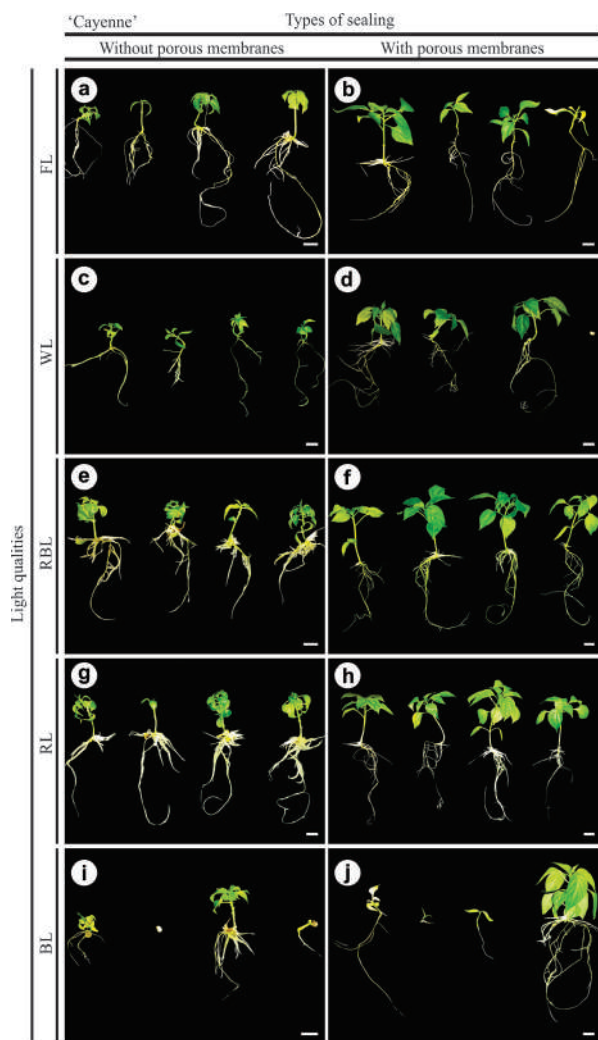


Figure 3. Plants of *Capsicum frutescens* cv. ‘Cayenne’ cultivated *in vitro* under different light qualities and types of sealing: Fluorescent (FL) without (a) and with porous membranes (b); White LED (WL) without (c) and with porous membranes (d); Red/blue LED (RBL) without (e) and with porous membranes (f); Blue LED (BL) without (g) and with porous membranes (h); Red LED (RL) without (i) and with porous membranes (j). Bars: 1 cm.

Photosynthetic pigments

By the analysis of variance, the chlorophyll *b* differed for the triple interaction between cultivars x light qualities x types of sealing. For the chlorophyll *a*, there was a significant difference for cultivar x light qualities and types

of sealing x light qualities. For carotenoids a significant difference was evidenced between cultivar x light qualities.

For chlorophyll *a* only 'Cayenne' in the blue LED was significantly higher compared to 'Espaguetinho' ($p < 0.05$). Light qualities within the cultivar, for 'Espaguetinho', the blue LED showed inferior results compared to the other light qualities, without differences for 'Cayenne' ($p < 0.05$) (Figure 6a). Still, for chlorophyll *a*, considering types of sealing within the light qualities, the treatment with membrane was superior compared to the treatment without membrane, only in the red/blue LED. For light qualities x types of sealing, the blue LED treatment was inferior to the other light qualities within the membrane treatment, while within the treatment without membrane, the

light qualities fluorescent and white LED were superior to the others (Figure 6b).

For 'Espaguetinho', cultivar x light qualities factorial was superior to 'Cayenne' for carotenoids only in red/blue and red LEDs. And for light qualities within the cultivar, plants grown under blue LEDs had less carotenoids than the other treatments in the 'Espaguetinho', while in the 'Cayenne' cultivar there was no significant difference among the light qualities (Figure 6c).

For the variable chlorophyll *b*, considering cultivar x light quality x types of sealing, the cultivar 'Espaguetinho', only the white LED treatment without membrane differed from the one with membrane; for 'Cayenne' there was no significant difference between types of sealing. In the types of sealing x cultivar x light qualities

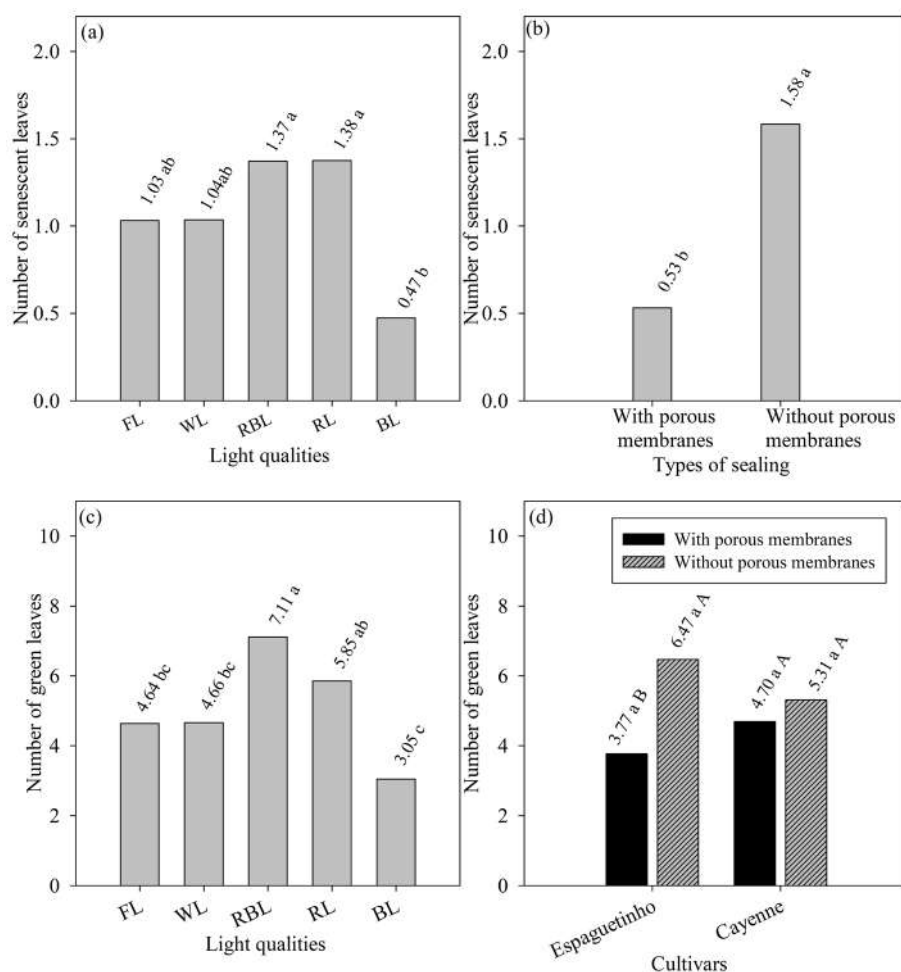


Figure 4. Growth variables of *Capsicum frutescens* cv. Cayenne and Espaguetinho, cultivated under different light qualities and types of sealing. Number of senescent leaves for the factor light qualities (a) and for the factor types of sealing (b); Number of green leaves for the factor light qualities (c) and for the interaction types of sealing and cultivars (d).^{*} Equal letters (figures a-c), do not differ by Tukey's test, 5% level; and lowercase letters equal in the same type of sealing (with or without membrane) and uppercase letters for the same cultivar ('Espaguetinho' or 'Cayenne') (figure d) do not differ from each other by the Tukey's test at 5% level.

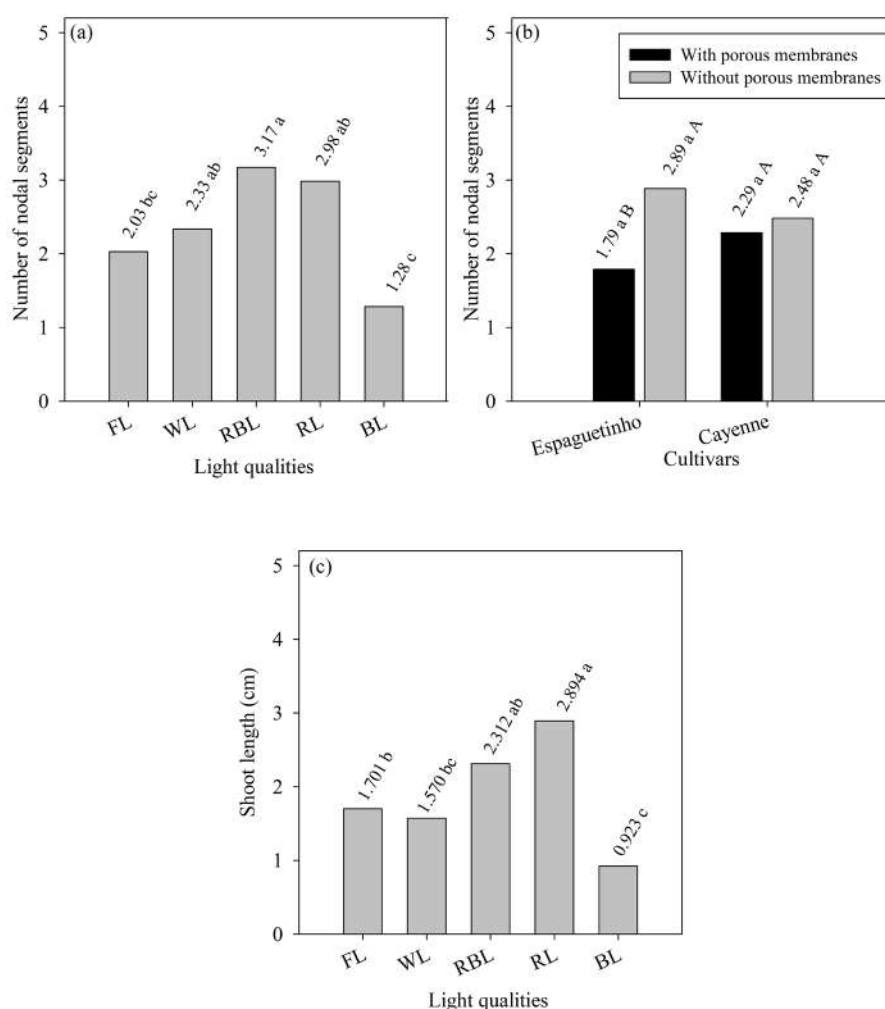


Figure 5. Growth variables of *Capsicum frutescens* cv. Cayenne and Espaguetinho cultivated under different light qualities and types of sealing. Number of nodal segments for the factor light qualities (a) and for the interaction types of sealing and cultivars (b); and shoot length for the light qualities factor (c). * Lowercase letters for light quality do not differ by Tukey's test, 5% level (a-c); Lowercase letters in the same type of sealing (with or without membranes) do not differ the cultivars between them, and uppercase letters in each cultivar do not differ for the type of sealing, by Tukey's test, 5% level (b).

Table I. Root length of *Capsicum frutescens* cultivars ('Espaguetinho' and 'Cayenne') cultivated under different light qualities and types of sealing.

	cv. Espaguetinho			cv. Cayenne	
	With porous membranes	Without porous membranes		With porous membranes	Without porous membranes
Light qualities	Root length (cm)				
Fluorescent lamp	2.98 aBβ	4.16 aAα		5.94 aAα	4,30 aABα
White LED	1.33 bBβ	5.35 aAα		5.24 aABα	3.69 aABα
Red/blue LED	4.34 aBα	5.50 aAα		6.92 aAα	4.88 aABα
Red LED	9.55 aAα	4.38 bAα		8.08 aAα	6.73 aAα
Blue LED	0.49 bBα	3.93aAα		1.71 aBα	2.01 aBα
CV (%)			37.41		

*Lowercase letters are equal in cultivars within each level of light qualities and types of sealing; uppercase letters are equal in light qualities within each level of cultivars and types of sealing; and Greek letters are equal in types of sealing within each level of cultivars and light qualities by Tukey's test, at 5% level.

Table II. Fresh weight of *Capsicum frutescens* cultivars ('Espaguetinho' and 'Cayenne') cultivated under different light qualities and types of sealing.

	cv. Espaguetinho			cv. Cayenne	
	With porous membranes	Without porous membranes		With porous membranes	Without porous membranes
Light qualities	Fresh weight (g)				
Fluorescent lamp	0.036 aBα	0.106 aBα		0.134 aAα	0.092 aBα
White LED	0.021 bBα	0.189 aBα		0.109 aAα	0.107 aBα
Red/blue LED	0.126 bABα	0.284 aAβ		0.180 bAα	0.436 aAα
Red LED	0.198 aAα	0.111 aABβ		0.114 bAα	0.254 aBα
Blue LED	0.093 aBα	0.099 aBα		0.037 aAα	0.099 aBα
CV (%)			10.18		

*Lowercase letters are equal in cultivars within each level of light qualities and types of sealing; uppercase letters are equal in light qualities within each level of cultivars and types of sealing; and Greek letters are equal in types of sealing within each level of cultivars and light qualities by Tukey's test, at 5% level.

Table III. Dry weight of *Capsicum frutescens* cultivars ('Espaguetinho' and 'Cayenne') cultivated under different light qualities and types of sealing.

	cv. Espaguetinho			cv. Cayenne	
	With porous membranes	Without porous membranes		With porous membranes	Without porous membranes
Light qualities	Dry weight (g)				
Fluorescent lamp	0.010 aAα	0.010 aAα		0.011 aAα	0.014 aBα
white LED	0.010 aAα	0.018 aAα		0.011 aAα	0.010 aBβ
Red/blue LED	0.014 aAα	0.018 aAβ		0.012 bAα	0.029 aAα
Red LED	0.016 aAα	0.013 aAα		0.004 bAβ	0.016 aBα
Blue LED	0.006 aAα	0.001 aAα		0.005 aAα	0.012 aBα
CV (%)			0.89		

*Lowercase letters are equal in cultivars within each level of light qualities and types of sealing; uppercase letters are equal in light qualities within each level of cultivars and types of sealing; and Greek letters are equal in types of sealing within each level of cultivars and light qualities by Tukey's test, at 5% level.

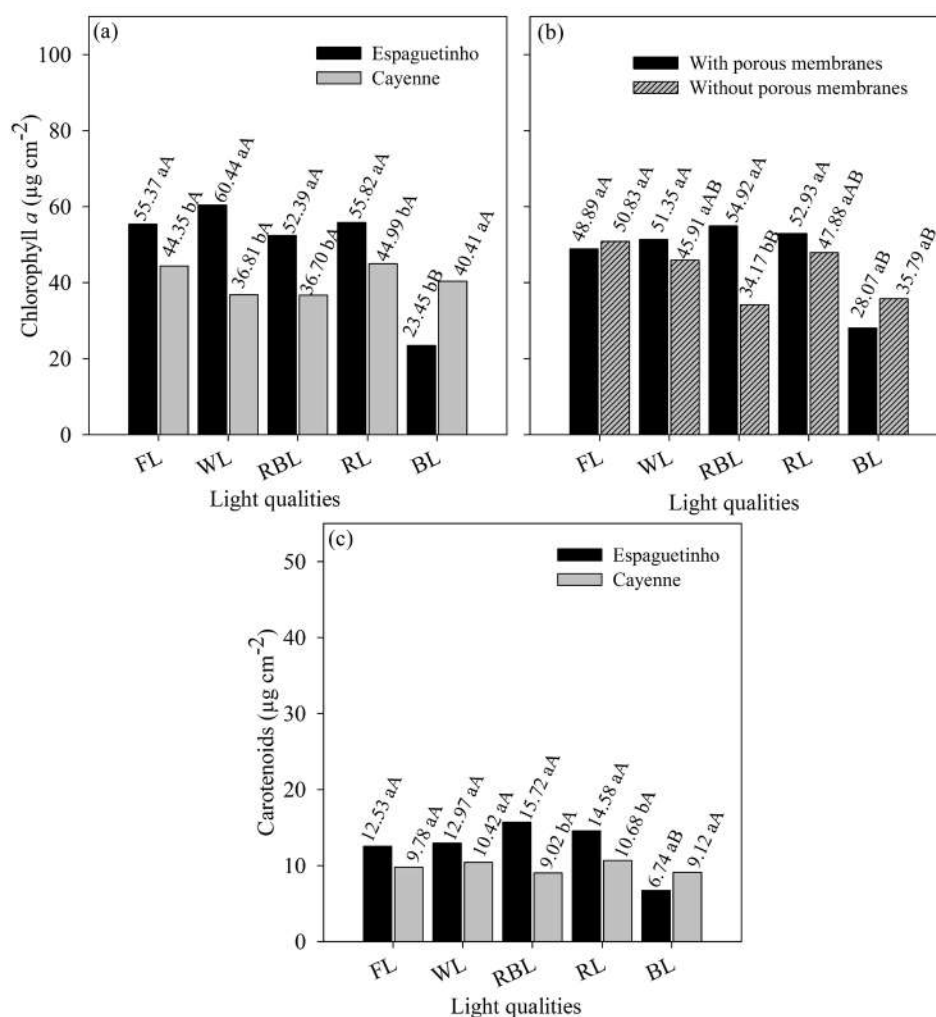


Figure 6. Photosynthetic pigments of *Capsicum frutescens* cv. Espaguetinho and Cayenne, cultivated under different light qualities and types of sealing. Chlorophyll a for the two cultivars in different light qualities (a); chlorophyll a for both types of sealing in different light qualities (b); Carotenoids for two cultivars in different light qualities (c). *Lowercase letters for the same cultivar and uppercase letters in the same light quality do not differ the cultivars from each other, by Tukey's test at 5% level (a and c). Lowercase letters for the same type of sealing and uppercase letters in the same light quality do not differ for the types of sealing, by Tukey's test, 5% level (b).

Table IV. Chlorophyll b contents of *Capsicum frutescens* cultivars ('Espaguetinho' and 'Cayenne') cultivated under different light qualities and types of sealing.

	cv. ‘Espaguetinho’			cv. ‘Cayenne’	
	With porous membranes	Without porous membranes		With porous membranes	Without porous membranes
Light qualities	Chlorophyll b (µg cm ⁻²)				
Fluorescent lamps	18.2585 aAα	18.6498 aBα		13.8718 aAα	9.5292 aAα
White LED	6.8423 bAα	36.7971 aAα		10.0560 aAα	6.0149 aAβ
Red/blue LED	11.0967 aAα	9.5425 aBα		8.2965 aAα	3.7168 aAα
Red LED	10.1240 aAα	7.8317 aBα		7.1692 aAα	4.7372 aAα
Blue LED	4.2678 aAα	8.5907 aBα		8.4236 aAα	8.3783 aAα
CV (%)			20.83		

*Lowercase letters are equal in cultivars within each level of light qualities and types of sealing; uppercase letters are equal in light qualities within each level of cultivars and types of sealing; and Greek letters are equal in types of sealing within each level of cultivars and light qualities by Tukey's test, at 5% level.

among the cultivars in the treatment with membrane, there was no significant difference between the light qualities; in the treatment without membrane only the white LED was superior in 'Espagueteinho', differing significantly from 'Cayenne' (Table IV).

DISCUSSION

This work describes *in vitro* germination, growth and development of *Capsicum frutescens* using different light qualities and gas permeable membranes. The use of gas-permeable membranes associated with red and red/blue LEDs, resulted in plants with significant differences in growth and morphophysiological parameters (Figures 1f, 1h, 3f, 3h, 4, 5 and 6). The germination and development of different species are affected by intrinsic characteristics of seeds or environmental factors such as light quality (Victório & Lage 2009); and, in this case, the red-light spectrum stimulates seed germination of several species (Figure 3g and 3h). Similar results were observed in the present work, in which the red LED with porous membrane presented greater percentage of germination for the cultivar 'Espagueteinho', and the red/blue LED without membrane for the 'Cayenne' cultivar (Figures 1h and 3h).

In this study, the use of porous membranes remarkably reduced the number of senescent leaves, a constraint in tissue culture systems due to either ethylene accumulation or the low nutrients absorption from the medium due low transpiration rates. Leaf abscission is a challenge for *in vitro* cultivation due to the accumulation of ethylene inside the culture environment and low gas exchange, which can generate physiological anomalies in the plants. Gas-permeable membranes have been used to provide the maintenance of CO₂ concentration, stimulate photosynthesis, lessen or inhibit the

negative effects of this ethylene, reduce the relative humidity, increase the transpiration and absorption of water and nutrients, stimulating the photoautotrophic development of plants *in vitro* (Rodrigues et al. 2011, Xiao et al. 2011). These gas exchanges allow ethylene releasing, which reduce the effect of leaf senescence (Rodrigues et al. 2011). This was also observed in this study when porous membranes were used (Figure 4a and 4b).

The progress of leaf senescence is influenced by physical and/or chemical factors in the growth conditions (e.g. temperature, precipitation and radiation). There are reports claiming that the plant regulates internal factors such as oxidative status and sugar levels to control senescence (Eldeen & Elgimabi 2017). Causin et al. (2006) observed that light quality affected oxidative metabolism in wheat leaves and senescence was delayed under blue light. In our work the blue light quality reduced leaf senescence in both cultivars (Figure 4a) however, plants produced under these conditions generated a reduced amount of green leaves (Figure 1i, 1j, 3i, 3j and 4c).

Plants are dependent on processes that involve the action of photoreceptors, which control plant development phases and are highly responsive to changes in light quality (Jensen et al. 2018, Braga et al. 2009). Wavelengths between 400 and 700 nm are required for the photosynthesis. These spectra were found in blue and red LED lamps, causing an increase in chlorophyll and carotenoids (Yeh & Chung 2009). In studies with *Lilium* sp., cultivar Pesarounder, red/blue LEDs promoted greater fresh and dry weight compared to fluorescent lamps (Lian et al. 2002). Rocha et al. (2010) also reported promising results on the use of red LEDs in photosynthetic pigment contents and a higher number of shoots in *Fragaria* x *Ananassa* plants cultured *in vitro*. These results corroborate with

the present work, demonstrating the superiority and efficiency of red/blue and blue LEDs for fresh and dry weight and photosynthetic pigments for both cultivars analyzed.

Regarding the photosynthetic pigments, Saldanha et al. (2012) observed an increase when plants of *Pfaffia glomerata* were cultivated in flasks with porous membranes. Likewise, in the present work, the use of porous membranes associated with red/blue LEDs favored the increase of total chlorophyll content (Figure 6a and 6b). Red LED also promoted a greater increase in chlorophyll content in *Ocimum basilicum* (Ahlman et al. 2017). Chlorophyll molecules absorb blue and red wavelengths more efficiently since they correspond to the light absorption spectra of chlorophyll and carotenoids, which improves the photosynthetic metabolism with characteristics associated to autotrophic growth in plants (Yeh & Chung 2009).

In this study, the red LED provided an increase in root length (Table I, Figures 1g, 1h, 3g, and 3h). Hung et al. (2016) also observed that red light was better for *in vitro* rooting of *Vaccinium corymbosum*. The recognition of light qualities by plants occurs by the phytochrome. The different light spectra increase active forms of phytochrome, inducing rooting (Godoi & Takaki 2005, Victório & Lage 2009). In fact, Pawłowska et al. (2018) observed that the red LED resulted in the formation of numerous adventitious roots with short lengths, which is beneficial during the transfer to *ex vitro* conditions.

As in the present work (Figure 5c), also observed a reduction in the growth of tomato seedlings cultivated under blue LED light compared to those cultivated under other light qualities. Pawłowska et al. (2018) also observed that the blue light strongly suppressed the growth of *Gerbera jamesonii*, causing a significant length reduction and shortening of petioles. This characteristic is related to the

inhibition of hypocotyl elongation, caused by cryptochrome signaling, due to the increased of blue light absorption by the phototropin in hypocotyl cells (Folta & Edgar 2001).

This work is the first report on the influence of light quality and sealing type in the *in vitro* germination, growth and development of two *Capsicum frutescens* cultivars. The use of porous membranes in the culture flasks is important to face frequent problems in conventional tissue culture such as leaf senescence. In general, the light quality provided by red and red/blue LEDs substantially improved the growth and development parameters of produced *in vitro* plants, which are superior to those cultured in fluorescent lamps commonly used in conventional cultivation, demonstrating that LEDs may be advantageous substituent in the cultivation of plants *in vitro*. Despite the cultivar 'Espagueteinho' showed higher chlorophyll a content than 'Cayenne', in general there were no differences in morphogenic responses between these cultivars, showing that the recommendations of *in vitro* culture conditions can be standardized for the two genotypes. Furthermore, the present study provides a basis for future researches and applications to anticipate the productive cycle of *Capsicum frutescens* under *ex vitro* conditions.

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Abbreviations

LED Light-emitting diodes
 FL Fluorescent lamps
 WL White LEDs

RL Red LEDs
 BL Blue LEDs
 RBL Red/blue LEDs
 CRD Completely randomized design

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Macrosporogenesis and development in *Bambusa tuldoidea* Munro and *Dendrocalamus asper* (Schult & Schult) Backer ex K. Heynek

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Abstract

Though the viability, morphology, and development of bamboo pollen have received considerable study, the female gametophyte has received comparatively less attention. In the present work, it was performed a detailed study of macrosporogenesis in three stages of flower development in *Dendrocalamus asper* and *Bambusa tuldoidea* looking at to elucidate the absence of caryopses formation. The gynoecium in three stages of floral development was analyzed using scanning electron microscopy and light microscopy techniques. Megasporeogenesis in *B. tuldoidea* begins at stage one of floral development with the formation of two megaspores, and megagametogenesis begins in the pre-anthesis with the formation of three abortive and one viable megaspores and ends in anthesis with the formation of a polygonum type embryonic sac. Megasporeogenesis in *D. asper* starts at stage two of floral development with the formation of the megaspore mother cell and megagametogenesis occurs between stages two and pre-anthesis, during anthesis, the caryopsis developed, and the torpedo-stage embryo collapsed. The studies of the developmental stages of *B. tuldoidea* and *D. asper* elucidated the absence of caryopsis formation, the first being related to the failure in the development of the pollen grain and in the second, the early inbreeding depression seems to be the reason for the non-formation of pollen grains caryopsis production. In addition, the absence of seeds negatively affects genetic variability, which is so important for genetic improvement programs and conservation of plant genetic resources.

Keywords Bamboo · Embryo collapse · Female gametophyte · Megagametogenesis

1 Introduction

Bamboos are large woody grasses (subfamily Bambusoideae, family Poaceae/Andropogoneae), comprising 1250 species within 75 genera spread over almost all continents except Europe and Antarctica (Williams et al. 1994; Scurlock et al. 2000).

Bamboos have a broad spectrum of uses, ranging from construction material, handicrafts, and food (Scurlock et al. 2000; Bonilla et al. 2010), making them a prime target for economic development (Costa et al. 2015). In Colombia and Ecuador, bamboo is a building material for both civil and agro-industries, promoting rural development (Guilherme et al. 2017).

Dendrocalamus asper (Schult. & Schult.) Backer ex K. Heynek, a species valued for its edible shoots, and *Bambusa tuldoidea* Munro, both native to China, is widely cultivated in America tropical and subtropical regions (Arya et al. 2001; Guerreiro and Lizarazu 2010).

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Despite their economic importance, bamboo reproduction remains poorly understood, likely due to the long reproductive cycles, which may range from 10 to 120 years of vegetative growth depending on the species. *B. tuldoidea* flowering is sporadic, with a time interval of 23 years of vegetative development. *D. asper*, on the other hand, blooms after 100 to 120 years (Nadgir et al. 1984; Ramanayake and Weerawardene 2003; Pilcher 2004; Guerreiro and Lizarazu 2010).

Sexual reproduction is an essential process because it is required to produce a new generation (Zhang et al. 2014). To successfully do so, female reproductive development must pass through several events, starting with megasporocyte specification to produce a functional megaspore (Reiser and Fischer 1993).

Most studies on reproductive biology emphasize the viability, morphology and development of the pollen grain in species such as *Chusquea subtessellata* Hitchc., *Olyra humilis* Nees, *Sucrea monophylla* Soderstr., *Dendrocalamus asper* (Schult & Schult) Backer ex K. Heynek, *Bambusa tuldoidea* Munro. (Horn and Clark 1992; Nakamura et al. 2010; Guimarães et al. 2017; Souza et al. 2020a, b). However, studies on the female gametophyte are scarce in the literature, and limited to some species such as *Bambusa tulda*, *B. multiplex*, *B. sinospinosa*, *Thyrsostachys siamensis*, *Arundinaria simonii* f. *heterophylla*, *Dendrocalamus giganteus*, *Neosinocalamus affinis* (Bhanwra et al. 2001; Lin and Ding 2013; Wang et al. 2015; Lin et al. 2018). Although gynoecium development in angiosperms shares common features different species show particular developmental characteristics (Silva et al. 2017). Such knowledge can provide a basis for future research concerning taxonomy, genetic improvement, phylogeny, seed production, and the consequent conservation of these species (Bhanwra et al. 1991; Plachno and Piotr Świątek 2009; Wang et al. 2015).

In the present study, we performed detailed studies on macrosporogenesis in three stages of flower development

of *Dendrocalamus asper* and *Bambusa tuldoidea*, aiming at to elucidate aspects of the non-production of caryopses.

2 Materials and methods

Plant material – *Bambusa tuldoidea* inflorescences were collected from mother plants located at Parque Cidade das Abelhas-Federal University of Santa Catarina UFSC (27° 32' 20.04" S, 48° 30' 10.87" O), in the city of Florianópolis, Santa Catarina–Brazil, in Jan 2017.

Dendrocalamus asper inflorescences were collected at Fazenda dos Bambus, belonging to the Jatobás Institute, in the city of Pardinho (23° 06' 37.33" S, 48° 22' 05, 60" O), São Paulo–Brazil, in Jun 2017.

The newly collected material was packed in plastic bags and packed in styrofoam boxes with ice for transport to the Laboratory of Developmental Physiology and Plant Genetics (LFDGV) where the analyses of floral development stages were carried out, and to the Central Laboratory of Electron Microscopy (LCME) from the Federal University of Santa Catarina (UFSC), Florianópolis, Santa Catarina, Brazil to perform the microscopy analysis.

Exsiccate of *B. tuldoidea* and *D. asper* were deposited at the Herbarium FLOR–UFSC, under the codes FLOR 63,000 and FLOR 64,899, respectively.

Floral development stages – One hundred inflorescences were collected to determine the stages of flower development. These were analyzed under a stereomicroscope (Olympus brand, model SZH10). The stages of *Bambusa tuldoidea* flower development were: stage one, pre-anthesis and anthesis (Table 1). The stages of *Dendrocalamus asper* flower development were: stage two of development, pre-anthesis and anthesis (stage six) (Table 2).

Table 1 Stages of *Bambusa tuldoidea* flower development based on Souza et al. 2020b

Development stage	Characteristics
Initial stage 1	Gynoecium and the androecium are protected by the lemma
Stage 2 (pre-anthesis)	The lemma begins to open
Stage 3 (anthesis)	Opening of the lemma and the palea and the beginning of anther exteriorization
Stage 4 (anthesis)	Anther externalization and dehiscence

Table 2 Stages of *Dendrocalamus asper* based on Souza et al. 2020a

Developmental stage	Characteristics
Initial stages 1 through 4	Gynoecium and the androecium are protected by the lemma
Stage 5 (pre-anthesis)	Opening of the lemma and exposure of the stigma
Stage 6 (anthesis)	Exteriorization of the anthers and stigma
Stage 7 (anthesis)	Senescence of the anthers and dispersal of pollen grains

Optical microscope analysis – Ten samples of gynoecium were collected in both species. In *Bambusa tuldoidea* the development stages were: stage one, pre-anthesis and anthesis stage, and in *Dendrocalamus asper* they were: stage two, pre-anthesis and anthesis (stage six).

The samples were fixed in paraformaldehyde solution (2.5%) in a 1:1 phosphate buffer (0.2 M, pH 7.3) for 24 h at 4 °C. After fixation, the material was washed three times in phosphate buffer for 30 min (Bouzon 2006) and dehydrated in a gradual ethanol series (Sanders et al. 1999). Subsequently, the samples were pre-infiltrated in LeicaTM historesin with 1:1 ethanol for 72 h and then infiltrated for an additional 72 h with LeicaTM historesin according to the manufacturer's instructions. The samples were sectioned in a rotating microtome (microTec brand, model CUT 4055), producing 5 µm thick cross-sectional and longitudinal sections. The sections were stained with toluidine blue, coomassie bright blue, and Lugol stain to identify starch (O'Brien et al. 1965; Fisher 1968; Johansen 1940). Observations and records were performed with the aid of a fluorescence microscope (Leica brand, model DM5500 B).

Analysis under scanning electron microscopy – Ten samples of gynoecium were collected in both species. In *Bambusa tuldoidea*, the inflorescence development included: stage one, pre-anthesis and anthesis stage (Fig. 1) and *Dendrocalamus asper* they were: stage two of development, pre-anthesis and anthesis (stage six).

The samples were fixed for 24 h in a solution containing 2.5% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.2) and 0.2 M sucrose. Subsequently, the samples were dehydrated in a gradual ethanol series (30%, 50%, 70% and 90% and twice in 100%) for 30 min in each (adapted from SCHMIDT et al. 2012). Samples were dried with CO₂ until the critical point with an EM-CPD-030 (Leica, Heidelberg, Germany). The dry samples were covered with 20 nm gold in a metallizer (Baltec, CED 030) (Schmidt et al. 2012) and observed in a Scanning Electron Microscope (SEM) (Jeol brand, model JSM-6390LV).

3 Results

Development of female structures in *Bambusa tuldoidea*

– The structure of *B. Tuldoidea* flowers is distinct at stage one of floral development: spikes consisted of several spikelets, each containing both male and female reproductive structures (Fig. 1a). Within the gynoecium, the cells in the outer wall to the ovary wall are bisected with juxtaposed cells (Fig. 1c). The ovarian parenchyma is large with evident nuclei. Similarly, the cells of the internal wall have evident nuclei but are shorter and elongated (Fig. 1b, c, e). An empty

space suggesting the ovarian locule, chalaza, and funicular region were also present in the ovary (Fig. 1b, c). The micropyle region was visible within the seminal rudiment. Internal and external integuments with two layers of cells suggest that the micropyle is bi-tegumented. The nucleus region stood out for being formed by larger cells and the formation of two megaspores distinguishable by their size and dense cytoplasm (Fig. 1b, c). The seminal rudiment was classified as orthotropic at this stage because it did not present any curvature and crassinucleate because more than one layer of cells in the nucleus surrounded the megaspore mother cell (Fig. 1b).

In the pre-anthesis stage, the androecium and the gynoecium in the spikelet were protected by the palea (Fig. 1d). The ovary developed a thicker outer and inner wall compared to the previous stage, and trichomes covered the epidermis. At this stage of development, the ovarian locule was most distinguishable due to the filling parenchyma of the ovary and the funicular (Fig. 1e, f). The seminal rudiment was connected to the ovary wall by the funicular and formed by the nucleus and the forming megaspores (Fig. e). The seminal rudiment showed increasing proximity to the micropyle close to the viable megaspore. Three abortive megaspores and the nucellar tissue were also evident (Fig. 1f).

At anthesis the spikelets showed a small opening with observable anthers (Fig. 1g–i). The gynoecium consisted of the obovate ovary, the short stylet and feathery stigmas (Fig. 1h). The ovary had a hair on its upper extremity that extended to the stylet (Fig. 1g, h). The internal wall, the parenchyma cells and the ovarian locule were all identifiable within the ovule (Fig. 1i). In the seminal rudiment, the nucleus and the embryonic sac were evident in the internal and external integuments (Fig. 1c). The embryonic sac was formed by a medium cell composed of two polar nuclei and a large vacuole (Fig. 1h, i). At the micropylar end, the oospheric apparatus showed the two synergids and the oosphere (Fig. 1i). The synergic nuclei and most of the cytoplasm were located in the micropylar zone and a single large vacuole was located in the chalazal zone (Fig. 1b–i).

Development of female structures in *Dendrocalamus asper*

– In *D. asper* in the early stages of floral development, it was possible to observe the development of the spike, composed of several closed pseudo-spikelets and protected by the lemma (Fig. 2a). In stage two of floral development, the gynoecium presented an ovary whose epidermis was bisected with juxtaposed cells that extended to the stylet (Fig. 2b, c). Internally, the ovarian parenchyma was composed of large cells whose nuclei were mostly peripheral. The internal ovarian wall was formed by smaller, elongated cells with evident nuclei. The chalaza region and the ovarian locule were observed (Fig. 2b, c). Seminal rudiment was classified as orthotropic because it has no curvature

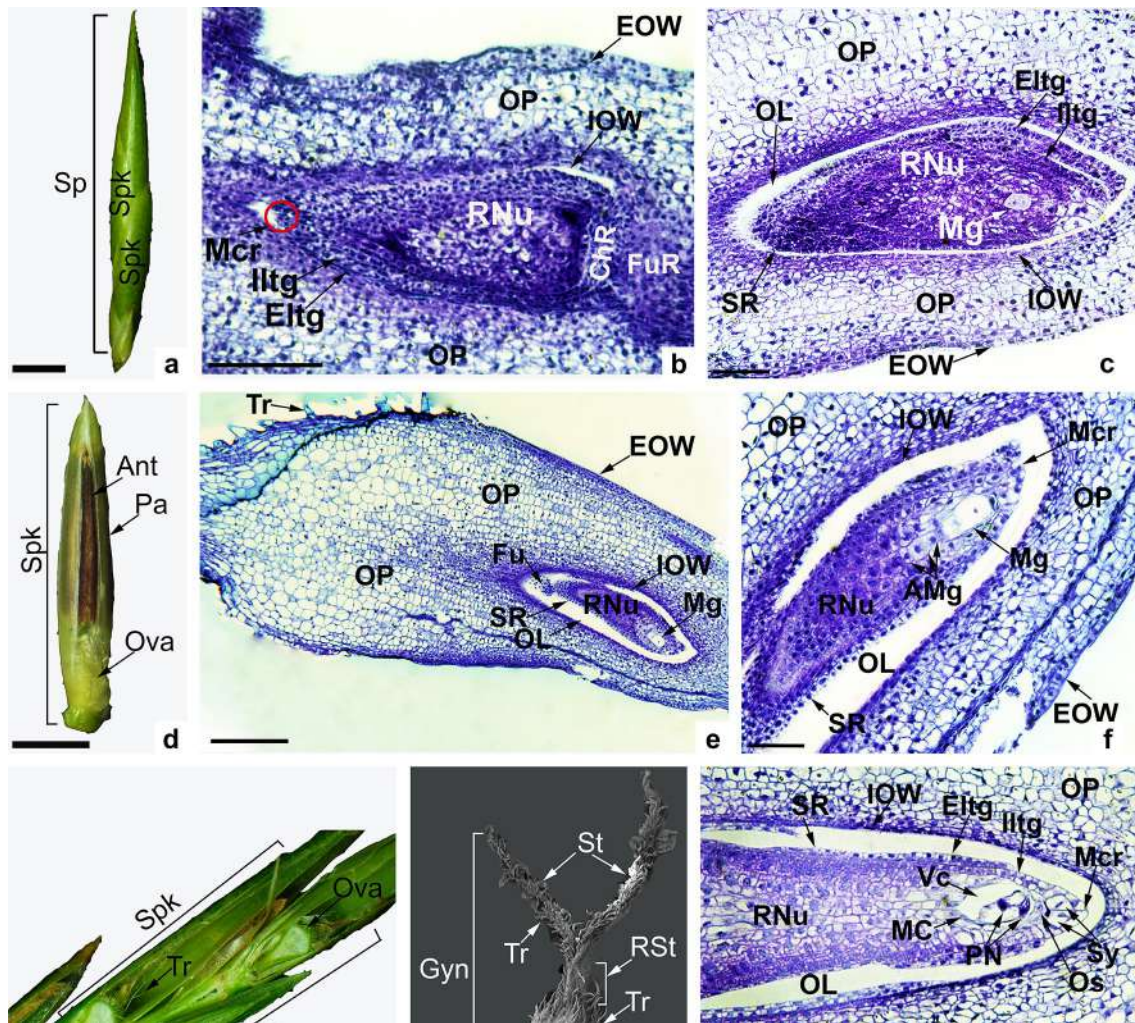


Fig. 1 Stereomicroscopy (a, d, g), optical (b, c, e, f, i) and scanning electron microscopies (h) of female reproductive structures in *Bambusa tul-doides*. **a, b, c:** stage one of floral development. **d, e, f:** pre-anthesis. **g, h, i:** anthesis. **a.** spikelet consisting of several spikelets. **b.** Histology of the ovary highlighting the external and internal walls, parenchymal cells with prominent nuclei and the funicular and chalazal regions. **c.** Seminal rudiment showing the micropylar region, the integuments, internal and external, and the nucellar region. Detail of the ovarian tissues delimiting an internal locule containing the seminal rudiment composed of the internal and external integuments, the nucleus, and two megaspores formation. **d.** Spikelet showing the anthers and ovaries protected by the palea. **e.** Overview of the tissues that make up the ovary, with trichomes that line the distal region, parenchymal cells bounded by internal and external walls. The seminal rudiment is inside the locule and connected to the ovary wall by the funicular, being formed by the nucellus and the forming megaspores. **f.** Detail of the ovary tissues, highlighting the seminal rudiment inside the locule and composed of the nucleus's micropylar region. It is possible to observe the three abortive megaspores and a viable megaspore. **g.** Spike formed by several spikelets, containing the ovary covered by trichomes. **h.** Gynoecium consisting of an obovate ovary, a short style and the bifid stigma. **i.** Ovary showing the inner wall, parenchyma cells and the ovarian locule. The seminal rudiment is composed of the internal and external integuments, the micropyle, the nucleus and the embryonic sac. Two synergids, the oosphere and, in the middle cell, the two polar nuclei and a large vacuole are present. Scale bar: a, d -0.5 cm; g -1 cm; b -100 μ m; c, f, i -50 μ m; and -200 μ m; h -500 μ m. Caption: AMg, abortive megaspores; Ant, anther; ChR, chalazal region; Eltg, external integument of the seminal rudiment; EOW, external ovarian wall; Fu, funicular; FuR, funicular region; Gyn, gynoecium; Iltg, internal integument of seminal rudiment; IOW, internal ovarian wall; MC, middle cell; Mcr, micropyle; Mg, megaspore; OL, ovarian locule; OP, ovarian parenchyma; Os, Oosphere; Ova, ovary; Pa, palea; PN, polar nuclei; RNu, Nucellar region; RSt, style region; Sp, spike; Spk, spiklet SR, seminal rudiment; St, bifid stigma; Sy, synergids; Vc, vacuole. (Optical microscopy: toluidine blue dye)

(Fig. 2b). In the seminal rudiment, internal and external integuments formed the bi-tegumented micropyle region. Both integuments had two layers of cells. The nucleus region stood out for its larger cells with evident nuclei, involving

the megaspore's mother cell, which characterizes the seminal rudiment as crassinucleate (Fig. 2b–d).

At pre-anthesis, the style was exterior of the pseudo-spikelets (Fig. 3a). The fruit and the seed were in the process

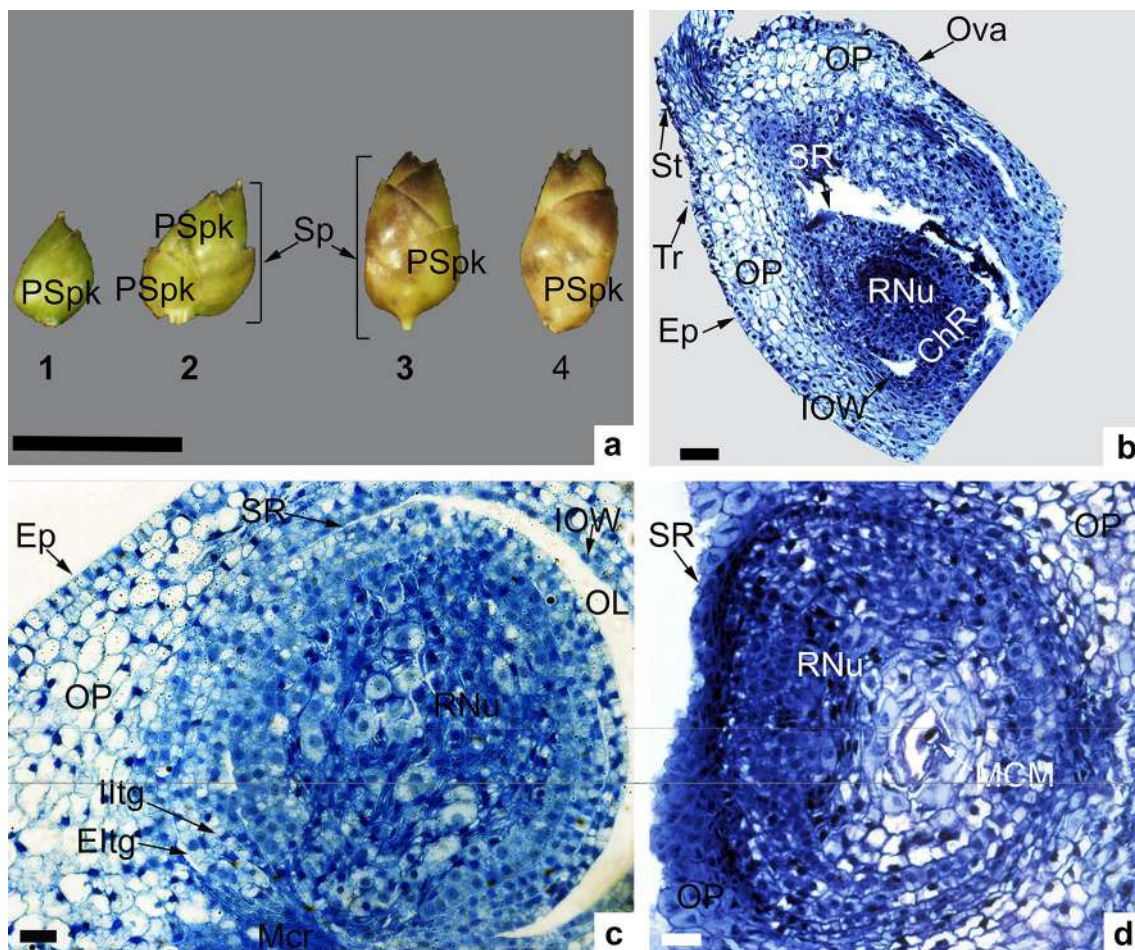


Fig. 2 Stereomicroscopy (**a**) and optical microscopy (**b–d**) of female reproductive structures in *Dendrocalamus asper*. **a.** early stages of floral development; **b, c, d:** stage two of development. **a.** Initial stages 1 to 4 showing spikes formed by the closed pseudo-spikelet (floret) and protected by the lemma. **b.** Longitudinal section of the ovary showing the chalazal region, the initial portion of the stylet, the epidermis with trichomes, the parenchyma and the internal ovarian wall. Seminal rudiment highlighting the nucellar region. **c.** Cross-section of the ovary showing the uniseriate epidermis, the ovarian locule, the parenchyma and the internal ovarian wall. The seminal rudiment consisted of internal and external integuments, the region of the micropyle, and nucellar. **d.** Cross-section of the ovary with seminal rudiment surrounded by ovarian tissues and showing the nucellar region and the megaspore mother cell. Scale bar: a -0.5 cm; b, c, d -50 μ m. Caption: ChR, chalaza region; Eltg, external integument of the seminal rudiment; Ep, ovarian epidermis; lltg, internal coat of seminal rudiment; IOW, internal ovarian wall; MCM, mother cell of the megaspore; MCR, micropylar region; OL, ovarian locule; OP, ovarian parenchyma; Ova, ovary; PSpk, pseudo-spikelet; RNu, nucellar region; Sp, spike; SR, seminal rudiment; St, stylet; Tr, trichome. (Optical microscopy- a, d: toluidine blue dye; c: coomassie bright blue)

of formation (Fig. 3b–d). The style showed a uniseriate epidermis with irregular cells, the epidermis of the fruit was biseriate, and both had trichomes (Fig. 3b). The pericarp was formed by the epidermis and parenchyma with large cells (Fig. 3b). The inner wall of the fruit consisted of small cells and evident nuclei (Fig. 3c). In the seed, the testa was formed by two layers of cells. The nucleus was reduced to two layers of cells when compared to the previous stage (Fig. 3b, c). More internally, the cellularized endosperm comprised several cells (Fig. 3c). The parenchyma, the fruit's inner wall and the micropyle region formed by layers of testa cells were observed (Fig. 4d). The nucleus showed large cells and the cellularized endosperm was composed of

few cells. The coenocytic endosperm appeared around the embryo in a unicellular stage (Fig. 4d).

4 Discussion

Macrosporogenesis in *Bambusa tuldoidea* – Sexual reproduction is an essential process because it is required to produce a new generation (Zhang et al. 2014). To successfully do so, female reproductive development must pass through several events, starting with megasporocyte specification to produce a functional megaspore (Reiser and Fischer 1993).

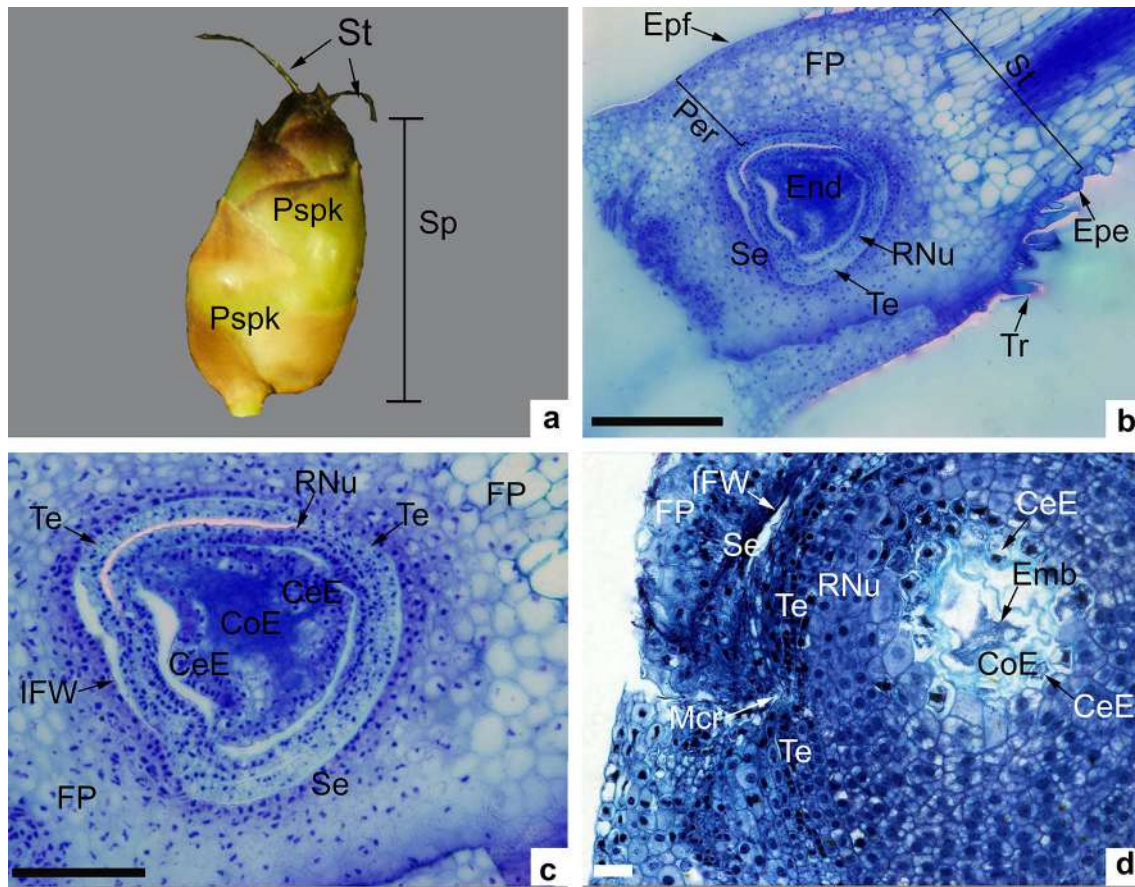


Fig. 3 Stereomicroscopy (**a**) and optical microscopy (**b–d**) of female reproductive structures in *Dendrocalamus asper* in pre-anthesis. **b, c**: Longitudinal section. **d**: Cross-section. **a**. Spike made up of several pseudo-spikelets with external stylus. **b**. Overview of the fruit showing the persistent style, the stylized epidermis, trichomes, the epidermis of the fruit and the pericarp region. Seed showing the testa, nucleus and endosperm. **c**. Fruit showing the parenchyma and the inner wall. Detail of the seed composed of the testa, nucellus, cellular endosperm and coenocytic endosperm. **d**. Ovary showing the parenchyma and the interned fruit wall. Seed composed of the testa, micropylar region, nucleus, cellularized endosperm, coenocytic endosperm and embryo. Scale bar: a -0.5 cm; B. 200 μ m; c. 100 μ m; d. 50 μ m. Caption: *CeE*, cellularized endosperm; *CoE*, coenocytic endosperm; *Emb*, embryo; *Epe*, stylized epidermis; *Epf*, epidermis of the fruit; *FP*, fruit parenchyma; *IFW*, internal fruit wall; *Mcr*, micropylar region; *End*, endosperm; *Per*, pericarp; *PSpk*, pseudo-spikelet; *RNu*, Nucellar region; *Se*, seed; *Sp*, spike; *St*, style; *Te*, seminal test; *Tr*, trichomes. (Optical microscopy: toluidine blue dye)

In *B. tulldoides* the mother cell of the megaspore undergoes the first meiosis in stage one of floral development (Fig. 2c). Subsequently, each of the formed megaspores undergoes another meiosis forming a linear tetrad of four megaspores during pre-anthesis (Drews et al. 1998). Eventually, all three non-functional megaspores degenerate completely, and the megaspore near the chalaza survives (Fig. 1f). Thus, the female gametophyte development of *B. tulldoides* is monosporic. This pattern of development is exhibited by many species, such as *Zea mays*, *Zea mays* subsp. *Parviglumis*, *Lolium multijlorum*, *Rostraria cristata*, *Digitaria abludens*, *Digitaria ciliar*, *Olyra humilis* Nees, *Sucrea monophylla* Soderstr, *Axonopus aureus* P. Beauv., *Paspalum polyphyllum* Nees ex Trin., *Chloris elata* Desv. and *Eragrostis solida* Nees (Bhanwra et al. 1991; Huang and Sheridan 1994;

Drews et al. 1998; Schneitz 1999; Nakamura et al. 2009; Wu et al. 2011).

Between pre-anthesis and anthesis, megagametogenesis occurs (Fig. 1h, i). The functional megaspore undergoes three mitosis cycles, producing a cell with eight nuclei: an oosphere, two synergids, the central cell with its two polar nuclei and three antipodes (Bhanwra et al. 1991; Drews et al. 1998). During anthesis, *B. tulldoides* produced an oosphere, two synergids and the central cell with its two nuclei (Fig. 1i). Synergistic cells have several structural specializations, they have a polarized cytoplasm; their nuclei and most of the cytoplasm is located in the micropylar pole; and a single large vacuole is located in the chalazal pole (Fig. 1i) Punwani and Drews (2008). The same authors report that these cells are specialized with many unique physiological

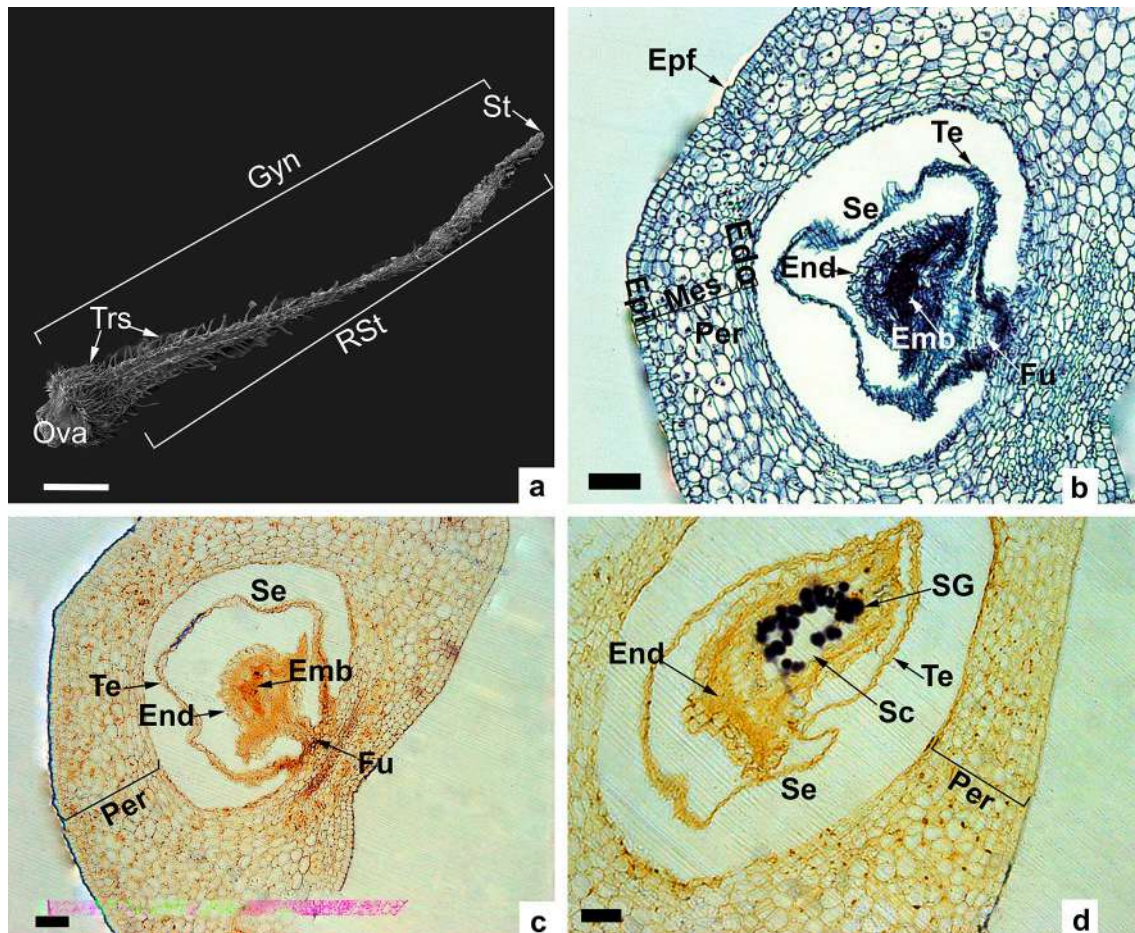


Fig. 4 Scanning electron microscopies (**a**) and optical (**b–d**) of female reproductive structures in *Dendrocalamus asper* at anthesis **b, c**: cross-section. **d**: longitudinal section. **a**. Ovate fruit showing persistent style and stigma. Trichomes cover all evident structures. **b**. Detail of the epidermis of the fruit and the pericarp with the external (epicarp), median (mesocarp) and internal (endocarp) regions. Seed connected to the fruit wall by the funicular, comprised of the testa, endosperm and torpedo-shaped embryo. **c**. Fruit with evident pericarp. Seed, attached to the fruit wall, showing the testa within the fruit's inner wall and the endosperm occupying a restricted space away from the testa and the embryo. **d**. Fruit showing the pericarp. Seed showing the testa, endosperm and scutellum with deposited starch granules. **a** - 500 μ m; **b, c, d** - 50 μ m. Caption: *Edo*, endocarp; *Emb*, embryo; *End*, endosperm; *Epf*, epidermis of the fruit; *Epi*, epicarp; *Fu*, funicular; *Gyn*, gynoeceium; *Mes*, mesocarp; *Ova*, ovary; *Per*, pericarp; *RSt*, style region; *Sc*, scutellum; *Se*, seed; *SG*, starch granules; *St*, stigma; *Te*, testa; *Trs*, trichomes. (Optical Microscopy—**b**: toluidine blue dye; **c, d**: lugol reagent)

and structural characteristics that facilitate the fertilization process. Synergistic cells secrete attractions that guide the pollen tube to the female gametophyte (Higashiyama et al. 2003) and probably contain factors that control pollen tube growth and gamete fusion (Weterings and Russell 2004).

The analytical procedures performed did not allow observe the antipodes. It is well known that many grasses have antipodes that undergo a series of mitotic divisions that lead to an ephemeral tissue composed of numerous cells reaching several hundred in bamboo (Diboll and Larson 1966). This type of development of female gametophytes is a modification of the Polygonum type that also occurs in *Zea mays* subsp. *parviglumis*, *Olyra humilis* Nees, *Sucrea monophylla* Soderstr, *Chloris elata* Desv. and *Eragrostis solida* Nees (Wu et al. 2011).

Despite the normal development of the embryonic sac, the production of cariopses in both species was not observed. However, a study by Souza et al. (2020b) found that *B. tuldoidea* developed abnormal pollen with signs of degradation of cytoplasmic organelles and cell membrane already in stage one of floral development. Moreover, when the embryonic sac is already fully developed in anthesis, the pollen grains present a fully degraded cytoplasm, leaving only the starch grains and an incomplete sporoderm (Table 3). Therefore, the non-production of caryopses is due to male sterility in this species.

Macrosporogenesis and formation of the caryopsis in *Dendrocalamus asper* – Megasporogenesis in *Dendrocalamus asper* begins at stage two of floral development, as observed

Table 3 Pollen grain development in the embryonic sac in *Bambusa tuldoidea*

Flower development stage	Microsporogenesis characteristics (Souza et al. 2020b)	Megasporogenesis and Megagametogenesis characteristics
Initial stage 1	Free microspores show cytoplasm retraction and rupture of the nuclear and mitochondrial membrane	Formation of two megaspores
Stage two (pre-anthesis)	Degenerate microspores, starch accumulated in numerous amyloplasts. Incomplete sporoderm	Presence of three abortive megaspores and one viable megaspore
Stage four (anthesis)	Degenerate microspores, starch accumulated in numerous amyloplasts. Incomplete sporoderm	Embryonic sac formed by a medium cell composed of two polar nuclei and a large vacuole, and the oospheric apparatus with two synergids and the oosphere

by a megaspore mother cell (Fig. 2d). Moreover, megagametogenesis occurs between stages two and pre-anthesis because the embryo is already present in pre-anthesis (Fig. 2b–d).

After successful fertilization, three semi-independent processes begin at developing the seed or caryopsis: the development of the embryo, endosperm and seed coat (Loch et al. 2004). In addition, the nucleus of endospermic and non-endospermic seeds partially degenerates to make room for the growth of the endosperm and embryo (Lu and Magnani 2018). In *D. asper*, a considerable reduction of the nucleus occurred between stages two and pre-anthesis (Fig. 2b–d and Fig. 3b–d). This pattern of reduction also occurs in *Sorghum bicolor*, in which five days after the fertilization event, the endosperm replaces a large part of the nucleus (Paulson 1969).

The endosperm of grasses follows a type of development called nuclear (or coenocytic) (Lopes and Larkins 1993; Olsen 2004). In this type of development, the initial triploid nucleus undergoes several divisions, but without forming a cell wall and cytokinesis, resulting in a syncytium formation. Subsequently, endosperm cellularization occurs (Nguyen et al. 2007; Sabelli and Larkins 2009). The syncytium grows extensively before being compacted and digested by the developing scutellum (Rudall and Dransfield 1989). In *D. asper*, the endosperm's cellularization begins around the embryo in the pre-anthesis and anthesis stages, where most of the endosperm is compacted and absorbed by the embryo

and scutellum (Fig. 3c, d and Fig. 4b–d). In *Melocalamus* sp. and *Ochlandra*, the endosperm becomes completely cellular when the embryo is in the globular stage (Gopal and Ram 1987). Moreover, as in *D. asper*, most of it is absorbed by the embryo during its development and differentiation.

The lining of the fruit and seeds develops from the ovary wall and the seminal rudiment's integuments, respectively (Desmastia et al. 2009). The outer integument of the seminal rudiment of *D. asper* degenerates between stages two and pre-anthesis (Fig. 2c and Fig. 3b–d). And according to Kraehmere and Baur (2019), the internal integument persists and develops, in some cases, into the seed coating. In the case of *D. asper*, the outer tegument originates from the testa and the wall of the ovary, the pericarp, and consists of three layers: the epicarp, mesocarp and endocarp (Fig. 3b–d). In *Bambusa tulda*, the outer tegument degenerates during megasporogenesis, and the internal tegument subsequently does so during the development of the embryonic sac (Gopal and Ram 1987).

All grasses, including bamboos, have a single-seeded fruit, in which the testa (seed coating) and pericarp fused to form a caryopsis (Rudall et al. 2005). Empty spaces between the pericarp and the testa, and between the testa and the embryo, are present in *D. asper* during anthesis (Fig. 4b–d). Embryo development stagnates during the torpedo stage at anthesis and becomes collapsed (Fig. 4b, c). This shows that caryopsis did not develop in this species in both species.

Table 4 Pollen and embryonic sac development in *Dendrocalamus asper*

Flower development stage	Microsporogenesis characteristics (Souza et al. 2020a)	Characteristics of megagametogenesis and caryopsis formation
Stage two	Free microspores with non-polarized nucleus. Presence of pore without exine	Formation of megaspore mother cell
Stage five (pre-anthesis)	Vacuolar microspore with polarized nuclei before first mitosis. Presence of the pore, operculum and ring	Presence of the pericarp, testa, reduced nucleus, cellularized and cenocytic endosperm and the embryo
Stage six (anthesis)	Mature trinucleated pollen containing one vegetative and two sperm nuclei	Presence of pericarp and pericarp. A large portion of the endosperm is absorbed by the scutellum. Stagnation and collapse of the embryo during torpedo stage

The low production or even the non-production of seeds or caryopses has been reported in several studies and attributed mainly to the non-viability of the pollen grain, to the physiological self-incompatibility and early inbreeding depression (Sage et al. 1999; Araujo et al. 2007; Souza et al. 2020a).

D. asper pollen from the same population showed normal development and were able to reach maturity (Souza et al. 2020a) (Table 4).

In physiological self-incompatibility, a self-recognition system based on genetics reduces the frequency of successful self-fertilization and increases heterosis, this mechanism is recognized mainly as a pre-zygotic barrier that can involve a pollen's failure to germinate in the stigma and/or impair the posterior growth of the pollen tube (De Nettancourt 1997). However, this is not the case for *D. asper*, as the embryonic sac is fertilizing, giving rise to a collapsed embryo (Fig. 3b, c).

Depression by early-acting inbreeding causes abortion of homozygous descent during embryo development due to the presence of deleterious recessive alleles (Husband and Schenke 1996; Araujo et al. 2007). Depression by early-acting inbreeding is a potential reason for the low production of seeds in *Brachiaria brizantha* (Araujo et al. 2007). This also seems to be the reason for the non-production of caryopsis in *D. asper*, as this species had intense flowering in all clumps in 2016, but in 2017, a fact that was followed by the authors in the city of Pardo (23° 06'37.33 "S, 48° 22'05.60" W), São Paulo–Brazil, when the material was collected, flowering was incipient and not all clumps bloomed.

As *D. asper* has a long period of vegetative growth, this population may have multiplied clonally through a complex system of underground rhizomes and these related individuals may have exchanged genetic material between themselves (Pohl 1991; Guilherme et al. 2017).

Another characteristic that reinforces this hypothesis is that the species undergoes protogyny to avoid inbreeding (Souza et al. 2020a). Protogyny is confirmed here in this study, since the complete development of the embryonic sac occurs before the development of the pollen grain, between stages two and pre-anthesis. The pollen grain, on the other hand, completes its development in anthesis (Table 4). Megasporogenesis in *Bambusa tuldoidea* begins at stage one of floral development, and megagametogenesis begins at pre-anthesis and ends at anthesis with the formation of the polygonum type embryonic sac. The non-production of caryopsis in *B. tuldoidea* cannot be explained by the development of the embryonic sac, being related to the failure to develop the pollen grain.

Megasporogenesis in *Dendrocalamus asper* starts at stage two of floral development, and megagametogenesis occurs between stages two and pre-anthesis. In the anthesis of this species, the beginning of the development of the caryopsis

and the collapse of the embryo were observed. Moreover, in *D. asper*, early inbreeding depression seems to be the reason for not producing caryopsis.

The studies of the developmental stages of *B. tuldoidea* and *D. asper* elucidated the absence of caryopsis formation, the first being related to the failure in the development of the pollen grain and in the second, the early inbreeding depression seems to be the reason for the non-formation of pollen grains caryopsis production. In addition, the absence of seeds negatively affects genetic variability, which is so important for genetic improvement programs and conservation of plant genetic resources.

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Authors' contribution Conception and design was carried out by PFDS, PR, CMRDS, and MPG. Material preparation, data collection and analysis were performed by PFDS, JR, and EMP. The first draft of the manuscript was written by PFDS, and all authors (PR, CMRDS, MPG, JR, EMP, TG) commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability The data are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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